



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(54) Title: BIOMATERIALS FORMED BY NUCLEOPHILIC ADDITION REACTION TO CONJUGATED UNSATURATED GROUPS</b><br><b>(54) Titre: BIOMATERIAUX FORMES PAR REACTION D'ADDITION NUCLEOPHILE A DES GROUPES NON SATURES CONJUGUES</b><br><br><b>(57) Abstract</b><br><p>The invention features polymeric biomaterials formed by nucleophilic addition reactions to conjugated unsaturated groups. These biomaterials may be used for medical treatments.</p><br><b>(57) Abrégé</b><br><p>L'invention concerne des biomatériaux polymères formés par réactions d'addition nucléophiles à des groupes non saturés conjugués. Ces biomatériaux peuvent être utilisés dans des traitements médicaux.</p> |                  |   |

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| <b>(21) International Application Number:</b> PCT/US00/02608<br><b>(22) International Filing Date:</b> 1 February 2000 (01.02.00)<br><b>(30) Priority Data:</b><br>60/118,093 1 February 1999 (01.02.99) US<br><b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b><br>US 60/118,093 (CIP)<br>Filed on 1 February 1999 (01.02.99)<br><b>(71)(72) Applicant and Inventor:</b> HUBBELL, Jeffrey, A.<br>[US/CH]; Unterdorfstrasse 22, CH-8126 Zumikon (CH).<br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> ELBERT, Donald<br>[US/CH]; Hochstrasse 9, CH-8044 Zurich (CH). LUTOLF,<br>Manthias [CH/CH]; Josefstr. 180, CH-8005 Zurich (CH).<br>PRATT, Alison [US/CH]; Forsterstr. 48, CH-8044 Zurich<br>(CH). SCHOENMAKERS, Ronald [NL/CH]; Zurichbergstr.<br>235, CH-8044 Zurich (CH). TIRELLI, Nicola [IT/CH];<br>Culmannstr. 34, CH-8006 Zurich (CH). VERNON, Brent<br>[US/CH]; Gumpenwiesenstr. 44, CH-8157 Dielsdorf (CH). | <b>(74) Agent:</b> BIEKER-BRADY, Kristina; Clark & Elbing, LLP, 176<br>Federal Street, Boston, MA 02110-2214 (US).<br><b>(81) Designated States:</b> AU, BR, CA, CN, CZ, GE, HU, ID, IL,<br>IS, JP, KR, MX, NO, NZ, PL, RO, RU, SG, TR, UA, US,<br>YU, European patent (AT, BE, CH, CY, DE, DK, ES, FI,<br>FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.</i> |   |
| <b>(54) Title:</b> BIOMATERIALS FORMED BY NUCLEOPHILIC ADDITION REACTION TO CONJUGATED UNSATURATED GROUPS<br><br><b>(57) Abstract</b><br><br>The invention features polymeric biomaterials formed by nucleophilic addition reactions to conjugated unsaturated groups. These biomaterials may be used for medical treatments.   |   |   |

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## Description

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BIOMATERIALS FORMED BY NUCLEOPHILIC ADDITION REACTION TO  
CONJUGATED UNSATURATED GROUPS

Cross Reference To Related Applications

This application claims priority to U.S.S.N. 60/118,093, filed February 1, 1999.

Background of the Invention

The present invention relates to biomaterials formed by nucleophilic addition reactions to conjugated unsaturated groups, and the uses of such biomaterials.

Synthetic biomaterials, including polymeric hydrogels can be used in a variety of applications, including pharmaceutical and surgical applications. They can be used, for example, to deliver therapeutic molecules to a subject, as adhesives or sealants, as tissue engineering and wound healing scaffolds, and as cell transplant devices.

While much progress has been made in the field of polymeric biomaterials, further developments must be made in order for such biomaterials to be used optimally in the body. For, example the formation of biomaterials in the presence of sensitive biological materials is difficult to achieve because the components of the biomaterials do not exhibit a high degree of self selectivity.

Summary of the Invention

New polymeric biomaterials, including polymeric hydrogels, have been developed for medical treatments. They are unique in their use of addition reactions between a strong nucleophile and a conjugated unsaturation, for polymerizing or cross-linking two or more components in a manner that can be accomplished in the presence of sensitive biological materials. This would include formation of biomaterials in the presence of

5 drugs, including proteins and DNA, formation of biomaterials in the presence of cells and  
cell aggregates, and also formation of biomaterials *in vivo* either within the body or upon  
10 the surface of the body. It is possible to form these biomaterials in the presence of  
sensitive biological materials because of the high self-selectivity of the addition reactions  
5 between strong nucleophiles and conjugated unsaturations, that are employed. The strong  
nucleophile of particular interest in the method described herein is the thiol.

15 In the formation of the biomaterial in the presence of the sensitive biological  
materials, two or more liquid components can be mixed together and react to form either  
an elastic solid, a viscoelastic solid (like a typical solid gel, for example, a gel like  
20 10 gelatin), a viscoelastic liquid (like a typical gel that can be induced to flow, for example, a  
gel like petroleum jelly), a viscoelastic liquid that is formed of gel microparticles (such as  
a Carbopol™ gel) or even a viscous liquid of a considerably higher viscosity than either  
25 of the two precursor components that are mixed together. The chemical conversion from  
the precursors to the final material is so selective that it can be carried out in the presence  
15 of the sensitive biological material, including the case when the biological material is the  
body itself.

30 A novel family of potentially highly biomimetic synthetic polymers has been  
developed. These polymers can: (i) be converted from liquid precursors to polymeric  
35 linear or cross-linked biomaterials either in the laboratory or *in situ* at a site of  
20 implantation; (ii) be hydrogels or more substantially non-swelling materials; (iii) present  
bioactive molecules that serve as adhesion sites, to provide traction for cell invasion; (iv)  
40 present bioactive molecules that serve as protease substrate sites, to make the material  
degrade in response to enzymes, such as collagenase or plasmin, which are produced by  
cells during cell migration; (v) present growth factor binding sites, to make the material  
25 interact with growth factors in a biomimetic manner, by binding them and then releasing  
them on cellular demand; and (vi) provide for the delivery of protein drugs by hydrolysis

5 or enzymatic degradation of groups contained within the backbone of the polymers that form the gel.

10 Accordingly, in a first aspect the invention features a method for making a biomaterial, involving combining two or more precursor components of the biomaterial under conditions that allow polymerization of the two components, where polymerization  
5 occurs through self selective reaction between a strong nucleophile and a conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic addition. The  
15 functionality of each component is at least two, and the biomaterial does not comprise unprocessed albumin. In addition, the conjugated unsaturated bond or group is not a  
20 maleimide or a vinyl sulfone.

In one embodiment of the first aspect of the invention, the components are selected from the group consisting of oligomers, polymers, biosynthetic proteins or peptides,  
25 naturally occurring peptides or proteins, processed naturally occurring peptides or proteins, and polysaccharides. The polymer may be poly(ethylene glycol), poly(ethylene  
15 oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(acrylic acid),  
30 poly(ethylene-co-acrylic acid), poly(ethyloxazoline), poly(vinyl pyrrolidone),  
poly(ethylene-co-vinyl pyrrolidone), poly(maleic acid), poly(ethylene-co-maleic acid),  
poly(acrylamide), or poly(ethylene oxide)-co-poly(propylene oxide) block copolymers.  
35 The peptide may comprise an adhesion site, growth factor binding site, or protease  
20 binding site.

In another embodiment, the components are functionalized to comprise a strong  
40 nucleophile or a conjugated unsaturated group or a conjugated unsaturated bond.  
Preferably the strong nucleophile is a thiol or a group containing a thiol. Preferably the  
conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or a  
25 vinylpyridinium, for example, 2- or 4-vinylpyridinium. In another embodiment,  
45 one component has a functionality of at least three.

5 In yet other embodiments of the first aspect of the invention, the method further  
comprises combining the precursor components with a molecule that comprises an  
adhesion site, a growth factor binding site, or a heparin binding site and also comprises  
10 either a strong nucleophile or a conjugated unsaturated bond or a conjugated unsaturated  
5 group. Preferably the strong nucleophile is a thiol or the conjugated unsaturated bond or  
conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or a vinyl  
15 pyridinium.

In still other embodiments of the first aspect of the invention, the biomaterial is a  
hydrogel. The biomaterial may also be degradable. The biomaterial may be made in the  
20 presence of sensitive biological molecules, or in the presence of cells or tissues. The  
10 biomaterial may also be made within or upon the body of an animal.

In still further embodiments of the first aspect of the invention, the method further  
25 comprises combining the precursor components with an accelerator prior to  
polymerization. The method may also further comprise mixing the precursor components  
15 with a component that comprises at least one conjugated unsaturated bond or conjugated  
unsaturated group and at least one amine reactive group. An additional component may  
30 also be applied to the cell or tissue surface site of polymerization, the additional  
component comprising at least one conjugated unsaturated bond or conjugated  
unsaturated group and at least one amine reactive group.

20 In a second aspect, the invention features a biomaterial formed by combining two  
or more precursor components of a biomaterial under conditions that allow  
polymerization of the two components, where polymerization occurs through self  
40 selective reaction between a strong nucleophile and a conjugated unsaturated bond or a  
conjugated unsaturated group, by nucleophilic addition. The functionality of each  
25 component is at least two, the biomaterial does not comprise unprocessed albumin, and  
45 the conjugated unsaturated bond or conjugated unsaturated group is not a maleimide or a



5 vinyl sulfone.

10 In one embodiment of the second aspect of the invention, the components are selected from the group consisting of oligomers, polymers, biosynthetic proteins or peptides, naturally occurring peptides or proteins, processed naturally occurring peptides  
5 or proteins, and polysaccharides. The polymer may be poly(ethylene glycol), poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(acrylic acid), poly(ethylene-co-acrylic acid), poly(ethyloxazoline), poly(vinyl pyrrolidone),  
15 poly(ethylene-co-vinyl pyrrolidone), poly(maleic acid), poly(ethylene-co-maleic acid), poly(acrylamide), or poly(ethylene oxide)-co-poly(propylene oxide) block copolymers.  
20 10 The peptide may comprise an adhesion site, growth factor binding site, or protease binding site.

25 In another embodiment of the second aspect of the invention, the components are functionalized to comprise a strong nucleophile or a conjugated unsaturated group or a conjugated unsaturated bond. Preferably the strong nucleophile is a thiol or a group  
15 containing a thiol. Preferably the conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or a vinylpyridinium, for example, 2- or 4-vinylpyridinium. In  
30 another embodiment, one component has a functionality of at least three.

35 In yet other embodiments of the second aspect of the invention, the method further comprises combining the precursor components with a molecule that comprises an  
20 adhesion site, a growth factor binding site, or a heparin binding site and also comprises either a strong nucleophile or a conjugated unsaturated bond or a conjugated unsaturated  
40 group. Preferably the strong nucleophile is a thiol or the conjugated unsaturated bond or conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or a vinyl  
pyridinium.

25 45 In still other embodiments of the first aspect of the invention, the biomaterial is a hydrogel. The biomaterial may also be degradable. The biomaterial may be made in the

5 presence of sensitive biological molecules, or in the presence of cells or tissues. The biomaterial may also be made within or upon the body of an animal.

10 In still further embodiments of the second aspect of the invention, the method further comprises combining the precursor components with an accelerator prior to  
5 polymerization. The method may also further comprise mixing the precursor components with a component that comprises at least one conjugated unsaturated bond or conjugated  
15 unsaturated group and at least one amine reactive group. An additional component may also be applied to the cell or tissue surface site of polymerization, the additional component comprising at least one conjugated unsaturated bond or conjugated  
20 10 unsaturated group and at least one amine reactive group.

25 In a third aspect, the invention features a method for delivering a therapeutic substance to a cell, tissue, organ, organ system, or body of an animal said method involving contacting the cell, tissue, organ, organ system or body with the biomaterial of  
30 15 the second aspect of the invention, wherein the biomaterial contains a therapeutic substance, whereby the therapeutic substance is delivered to the cell, tissue, organ, organ system, or body of an animal.

35 In one embodiment, the therapeutic substance is selected from the group consisting of proteins, naturally occurring or synthetic organic molecules, nucleic acid molecules, for example DNA or RNA, and a viral particle. In another embodiment, the therapeutic  
20 substance is a prodrug. In still another embodiment, the nucleic acid molecule is an antisense nucleic acid molecule.

40 In a fourth aspect, the invention features a method of regenerating a tissue, involving introducing a scaffold to a site, under conditions which permit cell ingrowth.. The scaffold may comprising the biomaterial of the second aspect of the invention.

25 45 In embodiments of the fourth aspect of the invention, the scaffold has been pre-seeded with cells. The tissue may be selected from the group consisting of bone, skin,

5 nerve, blood vessel, and cartilage.

10 In a fifth aspect, the invention features a method of preventing adhesions, thrombosis, or restenosis, involving contacting a site with the biomaterial precursor components of the second aspect of the invention; and polymerizing the components at  
5 the site.

15 In a sixth aspect, the invention features a method of sealing a fluid or gas flow, said method comprising the steps of contacting a site within the body of an animal with the biomaterial precursor components of the second aspect of the invention, which may further comprise a component that includes at least one conjugated unsaturated bond or  
20 10 conjugated unsaturated group and a least one amine reactive group; and polymerizing the components at the site.

25 In preferred embodiments of the sixth aspect of the invention, the site is a lung, blood vessel, skin, dura barrier, or intestine.

30 In a seventh aspect, the invention features a method of encapsulating a cell or tissue, involving combining the precursor components of a biomaterial with a cell or  
15 tissue; and polymerizing the components, where polymerization occurs through self selected reaction between a strong nucleophile and a conjugated unsaturated bond or a conjugate unsaturated group, and where the cell or tissue is encapsulated by the  
35 polymerized biomaterial.

40 In an eighth aspect, the invention features a method for making a biomaterial, involving combining two or more precursor components of the biomaterial under conditions that allow polymerization of the two components, where the polymerization  
25 occurs through self selective reaction between an amine and a conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic addition, wherein the functionality of each component is at least two, and wherein the biomaterial does not  
45 comprise unprocessed albumin, and the unsaturated bond or group is not a maleimide or a

5 vinyl sulfone.

10 In a ninth aspect, the invention features a biomaterial, formed by combining two or more precursor components of the biomaterial under conditions that allow polymerization of the two components, where the polymerization occurs through self selective reaction  
5 between an amine and a conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic addition, wherein the functionality of each component is at least two, and  
15 wherein the biomaterial does not comprise unprocessed albumin, and the unsaturated bond or group is not a maleimide or a vinyl sulfone.

20 By "biomaterials" is meant material which is intended for contact with the body, either upon the surface of it or implanted within it. Preferably, the biomaterial is formed by a conjugate addition reaction between a strong nucleophile and a conjugated  
10 unsaturation.

25 As used herein, the words "polymerization" and "cross-linking" are used to indicate a linking of multiple precursor component molecules to result in a substantial increase in molecular weight. "Cross-linking" further indicates branching, typically to  
15 yield a polymer network.

30 By "self selective" is meant that a first precursor component of the reaction reacts much faster with a second precursor component of the reaction than with other compounds present in the mixture at the site of the reaction, and the second precursor  
35 component reacts much faster with the first precursor component than with other compounds present in the mixture at the site of the reaction. The mixture may contain other biological materials, for example, drugs, peptides, proteins, DNA, cells, cell  
40 aggregates, and tissues. As used herein, a strong nucleophile preferentially binds to a conjugated unsaturation, rather than to other biological compounds, and a conjugated  
25 unsaturated group preferentially binds to a strong nucleophile rather than to other biological compounds.

5 When the highest degree of self selectivity is desired in the methods of the invention, a thiol is the nucleophile of choice. When the highest level of selectivity is not  
10 required in the methods of the invention, an amine may be used as the strong nucleophile. Conditions utilized to complete the self selective reaction of the present invention can be  
5 altered to increase the degree of self selectivity, as provided herein. For example, if an amine is used as the strong nucleophile in the formation of a biomaterial by selection of  
15 an amine with a low pK, and the final precursor solution to be polymerized is formulated such that the pH is near the pK, the reaction of the unsaturation with the provided amine is favored and thus self selectivity is achieved.

20 10 By "strong nucleophile" is meant a molecule which is capable of donating an electron pair to an electrophile in a polar-bond forming reaction. Preferably the strong nucleophile is more nucleophilic than H<sub>2</sub>O at physiologic pH. Examples of strong  
25 nucleophiles are thiols and amines.

15 A thiol is the preferred strong nucleophile to be used in the present invention, as it exhibits high self-selectivity. Very few sterically accessible thiols are present in proteins that are found outside cells. Amines may also be useful and self-selective especially  
30 when the biomaterial-forming reaction is conducted in the presence of sensitive biological molecules that do not bear amines, for example, many drugs.

35 20 By "conjugated unsaturated bond" is meant the alternation of carbon-carbon, carbon-heteroatom or heteroatom-heteroatom multiple bonds with single bonds, or the linking of a functional group to a macromolecule, such as a synthetic polymer or a  
40 protein. Such bonds can undergo addition reactions.

45 25 By "conjugated unsaturated group" is meant a molecule or a region of a molecule, containing an alternation of carbon-carbon, carbon-heteroatom or heteroatom-heteroatom multiple bonds with single bonds, which has a multiple bond which can undergo addition reactions. Examples of conjugated unsaturated groups include, but are not limited to

5 acrylates, acrylamides, quinones, and vinylpyridiniums, for example, 2- or 4-  
vinylpyridinium.

10 By "substantially pure peptide," "substantially pure polypeptide", or "substantially  
pure protein" is meant a polypeptide that has been separated from the components that  
5 naturally accompany it. As used herein the terms peptide, polypeptide, and protein are  
used interchangeably. Typically, the polypeptide is substantially pure when it is at least  
15 60%, by weight, free from the proteins and naturally-occurring organic molecules with  
which it is naturally associated. Preferably, the polypeptide is at least 75%, more  
preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A  
20 substantially pure polypeptide of interest may be obtained, for example, by extraction  
from a natural source (e.g., a cell, cell aggregate, or tissue) by expression of a  
recombinant nucleic acid encoding the desired polypeptide, or by chemically synthesizing  
25 the protein. Purity can be assayed by any appropriate method, for example, by column  
chromatography, polyacrylamide gel electrophoresis, agarose gel electrophoresis, optical  
15 density, or HPLC analysis.

30 A protein is substantially free of naturally associated components when it is  
separated from those contaminants which accompany it in its natural state. Thus, a  
protein which is chemically synthesized or produced in a cellular system different from  
35 the cell from which it naturally originates will be substantially free from its naturally  
associated components. Accordingly, substantially pure polypeptides include those  
20 derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

40 By "purified nucleic acid" is meant a nucleic acid molecule that is free of the  
genes which, in the naturally-occurring genome of the organism from which the nucleic  
acid of the invention is derived, flank the gene. The term therefore includes, for example,  
45 25 a recombinant DNA which is incorporated into a vector; into an autonomously replicating  
plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists

5 as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR  
or restriction endonuclease digestion) independent of other sequences. It also includes  
10 recombinant DNA which is part of a hybrid gene encoding additional polypeptide  
sequence.

5 By "functionalize" is meant to modify in a manner that results in the attachment of  
a functional group or moiety. For example, a molecule may be functionalized by the  
15 introduction of a molecule which makes the molecule a strong nucleophile or a  
conjugated unsaturation. Preferably a molecule, for example PEG, is functionalized to  
become a thiol, amine, acrylate, or quinone.

20 10 Proteins in particular may also be effectively functionalized by partial or complete  
reduction of disulfide bonds to create free thiols.

25 By "functionality" is meant the number of reactive sites on a molecule. As used  
herein, the functionality of a strong nucleophile and a conjugated unsaturation will each  
be at least two. Mixing two components, for example, a strong nucleophile and a  
15 conjugated unsaturation, with functionalities of two each will result in a linear polymeric  
biomaterial, and the mixing to two components with functionalities of at least two each,  
30 one of the components having a functionality of more than two, will result in a cross-  
linked biomaterial.

35 20 By "adhesion site" is meant a peptide sequence to which a molecule, for example,  
an adhesion-promoting receptor on the surface of a cell, binds. Examples of adhesions  
sites include, but are not limited to, the RGD sequence from fibronectin, and the YIGSR  
sequence from laminin. Preferably adhesion sites are incorporated into the biomaterial of  
40 the present invention.

25 By "growth factor binding site" is meant a peptide sequence to which a growth  
factor, or a molecule(s) which binds a growth factor binds. For example, the growth  
45 factor binding site may include a heparin binding site. This site will bind heparin, which

5 will in turn, bind heparin-binding growth factors, for example, bFGF, VEGF, BMP, or TGF $\beta$ .

10 By "protease binding site" is meant a peptide sequence which is a substrate for an enzyme.

5 By "antisense nucleic acid" is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand gene encoding a protein of interest. Preferably, the antisense nucleic acid is capable of decreasing the biological activity of said protein of interest when present in a cell. Preferably, the decrease is at least 10%, relative to a control, more preferably, 25%, and most preferably, 100%.

20 10 By "biological activity" is meant functional events mediated by a protein of interest. In some embodiments, this includes events assayed by measuring the interactions of a polypeptide with another polypeptide. It also includes assaying the effect which the protein of interest has on cell growth, differentiation, death, migration, adhesion, interactions with other proteins, enzymatic activity, protein phosphorylation or dephosphorylation, transcription, or translation.

30 By "sensitive biological molecule" is meant a molecule that is found in a cell, or in a body, or which can be used as a therapeutic for a cell or a body, which may react with other molecules in its presence. Examples of sensitive biological molecules include, but are not limited to, peptides, proteins, nucleic acids, and drugs. In the present invention 35 20 biomaterials can be made in the presence of sensitive biological materials, without adversely affecting the sensitive biological materials.

40 As used herein, by "regenerate" is meant to grow back a portion, or all of, a tissue. For example, the present invention features methods of regenerating bone following trauma, tumor removal, or spinal fusion, or for regenerating skin to aid in the healing of 25 45 diabetic foot ulcers, pressure sores, and venous insufficiency. Other tissues which may be regenerated include, but are not limited to, nerve, blood vessel, and cartilage tissue.



By "cell transplantation" is meant transplanting a cell, cell aggregate, or tissue into a subject. The biomaterial of the present invention can be used to isolate transplanted cells, cell aggregates, or tissues in the subject from the subject's defense system, while allowing the selective transport of molecules required for normal cell function.

#### Brief Description of the Drawings

Fig. 1 is a graph of the effect of changing the amino acid residues adjacent to cysteine on the rate of conjugate addition on acrylates (PEG-acrylate).

Fig. 2 is a schematic representation of a conjugate addition reaction, used as a model to study kinetics of a thiol (on cysteine) addition to the acrylate on PEG diacrylate.

Fig. 3 is a graph showing the effect of pH on the addition reaction between a thiol (on cysteine) and PEG diacrylate.

Fig. 4 is a graph of the effect of different PEGDA contents on the absorbance per mole of reagent, the average extinction coefficient (i.e., absorbance divided by the sum of the PEGDA and cysteine concentration; this sum is kept constant to  $2.5 \times 10^{-3}$  M).

Fig. 5 is a graph showing the effect the steric influence of groups near the site of the conjugated unsaturation has on the reaction between a thiol (on cysteine) and an acrylate, crotonoate, or dimethylacrylate of an accordingly functionalized PEG.

Fig. 6 is a graph showing the effect of the incorporation of an RGD peptide sequence into hydrogels of the present invention on cell adherence and spreading.

Fig. 7 is a graph showing the release of myoglobin from hydrogel-embedded collagen (Helistat) sponges. Note that at day 14, plasmin was added to the materials and this lead to the release of more myoglobin from the plasmin-sensitive hydrogels.

Fig. 8 is a strain-stress curve for a 75% solid gel prepared in an aqueous system. The gels were prepared using pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 at 75% solid in phosphate buffered saline at pH 9.0. The gels showed

5 approximately 37% deformation and 2 MPa Ultimate strength when submitted to compressive loads.

10 Fig. 9 shows stress-strain curves for a 75% solid gel prepared in an aqueous system with various contents of pentaerythritol triacrylate replacing the PEG diacrylate 570. The gels were prepared using pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 and pentaerythritol triacrylate at 75% solid in phosphate buffered saline at pH 9.0. The gels showed that the stiffness of the gel was manipulated by the content of the hydrophobic triacrylate.

15 Fig. 10 is a graph showing the effect of the addition of inorganic particles or surfactants to the gels on the ultimate strength of the gels. Gels prepared in the aqueous system at 75% solid (75% solid gels) were compared to those in which BaSO<sub>4</sub> was added at 10%, or when a surfactant, sorbitan monooleate (Emulsion), was added at 1%. Gel obtained from precursors pre-reacted were also compared to gels obtained by the pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 precursors (Pre-reacted precursors).

20 Fig. 11 is a graph showing the effect of the addition of inorganic particles or surfactants to the gels on the stiffness of the gels. Gels prepared in the aqueous system at 75% solid (75% solid gels) were compared to those in which BaSO<sub>4</sub> was added at 10%, or when a surfactant, sorbitan monooleate (Emulsion), was added at 1%. Gels obtained from precursors pre-reacted were also compared to gels obtained by the pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 precursors (Pre-reacted precursors).

25 Fig. 12 is a stress-strain curve for a gel prepared in an aqueous system loaded with fumed silica (14 nm). The gels were prepared using pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 in phosphate buffered saline at pH 9.0, reinforced with fumed silica particles (14 nm).

Fig. 13 shows a stress-strain curve for a 10% solid gel prepared in a N-methyl pyrrolidone/PEG 400 cosolvent. The gels were prepared using pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 at 10% solid in N-methyl pyrrolidone/PEG 400.

Fig. 14 shows elastic and complex moduli ( $G'$  and  $G''$ ) for pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570. Pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 were mixed with a 1 SH to 1 C=C ratio without phosphate buffered saline pH 9.0 buffer. The mixture was vortexed and then the elastic and complex moduli were determined with time by rheology.

Fig. 15 shows elastic and complex moduli ( $G'$  and  $G''$ ) at 37°C for pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 activated with phosphate buffered saline at pH 9.0. Pentaerythritol tetrakis (3-mercaptopropionate) and PEG diacrylate 570 were mixed with a 1 SH to 1 C=C ratio and phosphate buffered saline pH 9.0 was added. The mixture was vortexed and then the elastic ( $\diamond$ ) and complex ( $\blacksquare$ ) moduli were determined with time by rheology.

### Detailed Description

#### I. *In vivo* Synthesis or Application of Biomaterials

##### **The chemical reaction system used for biomaterial formation**

A novel chemical reaction scheme has been developed by which to polymerize or cross-link (the words are used as synonyms herein) two or more precursor components of a biomaterial *in situ* or in the presence of sensitive biological materials in a very self-selective manner. Commonly, two precursor components are mixed together. These two precursor components are self-selective in their reaction rates (i.e., a first precursor component reacts much faster with a second precursor component than with other

5 components in the sensitive biological material and the second precursor component  
reacts much quicker with the first precursor component than with other components in the  
sensitive biological material). When both of these precursor components have a  
10 functionality of at least two, and when one of them has a functionality greater than two,  
5 the system will self-selectively react to form a cross-linked biomaterial. The word  
'functionality' is used here in the sense used in polymer science (i.e., the number of  
15 reactive sites). Thus, mixing two components with functionalities of two each will result  
in a linear polymeric biomaterial, and the mixing to two components with functionalities  
of at least two each, one of the components having a functionality of more than two, will  
20 result in a cross-linked biomaterial.

10 When both of the precursor components have a functionality of two, a linear  
polymeric biomaterial will result. Both situations can be useful. In cross-linked  
25 biomaterials, the components can be very hydrophilic and the overall material can yet  
remain as an intact solid, not dispersing throughout the body. If such a non-dispersing  
15 system is desired for a linear polymeric biomaterial, it is useful if at least one precursor  
component be hydrophobic, such that the resulting biomaterial also be insoluble in water  
30 or body fluids. Other approaches are also possible, for example, when the two precursor  
components otherwise interact to become insoluble, or, when one or both precursors  
35 respond to pH, temperature or other stimuli to become more or less soluble, or when one  
20 precursor component is a polycation and the other precursor component is a polyanion, or  
when one precursor component strongly hydrogen bonds to the other.

40 The chemical reaction system of the present invention makes use of addition  
reactions, in which one component possesses a strong nucleophile and the other  
component possesses a conjugated unsaturation, or a conjugated unsaturation. Of  
45 25 particular interest in this invention as strong nucleophiles are thiols. Preferably, the  
system makes use of conjugate addition reactions between a thiol and a conjugated

5 unsaturation (e.g., an acrylate or a quinone). This reaction system can be made to be self-selective, meaning substantially unreactive with other chemical groups found in most  
10 sensitive biological compounds of interest (most drugs, peptides, proteins, DNA, cells, cell aggregates, and tissues). It is particularly useful when one or both of these  
5 components is part of a polymer or oligomer, however other possibilities are also indicated herein.

15 Proteins contain the amino acid cysteine, the side chain of which terminates in a thiol. In spite of this, there are very few free thiols within the protein: most proteins  
20 contain an even number of cysteine residues, and these are then paired and form disulfide cross-links between various regions of the protein. Some proteins contain an odd number  
10 of cysteine residues and most of these are present as disulfide linked dimers, again resulting in no free thiol residues being present in the native protein. Thus, there are very  
25 few free thiols in proteins. Some important electron transferring molecules, such as glutathione, contain a free thiol, but these molecules are generally restricted in their  
15 spatial location to the inside of a cell. Accordingly, conjugated unsaturated structures presented outside the cell will be substantially unreactive with most proteins at near-  
30 physiological conditions. Amines are also nucleophiles, although not as good a nucleophile as thiols. The pH of the reaction environment is important in this  
35 consideration. In particular, unprotonated amines are generally better nucleophiles than protonated amines. At physiological pH, amines on the side chain of lysine are almost  
20 exclusively protonated, and thus not very reactive. The alpha amine of the N-terminus of peptides and proteins has a much lower pK than the side chain epsilon amine;  
40 accordingly, at physiological pH it is more reactive to conjugate additions than are the epsilon amines of the lysine side chain.

25 Notwithstanding, the thiol is substantially more reactive than the unprotonated amine. As stated, the pH is an important in this consideration: the deprotonated thiol is

5 substantially more reactive than the protonated thiol. In conclusion, the addition reactions  
involving a conjugated unsaturation, such as an acrylate or a quinone, with a thiol, to  
10 convert two precursor components into a biomaterial will often be best carried out  
(meaning fastest, most self-selective) at a pH of approximately 8, where most of the thiols  
5 of interest are deprotonated (and thus more reactive) and where most of the amines of  
interest are still protonated (and thus less reactive). When a thiol is used as the first  
15 component, a conjugate structure that is selective in its reactivity for the thiol relative to  
amines is highly desirable.

20 If the conjugated structures are kept outside of cells, there are very few reactive  
10 nucleophiles with which to react to induce toxicity. One can typically accomplish this  
spatial restriction by making the conjugated component be of high molecular weight, be  
hydrophilic, or both.

25 Polyethylene glycol (PEG) provides a very convenient building block. One can  
readily purchase or synthesize linear (meaning with two ends) or branched (meaning  
15 more than two ends) PEGs and then functionalize the PEG end groups to introduce either  
a strong nucleophile, such as a thiol, or a conjugated structure, such as an acrylate or a  
30 quinone. When these components are either mixed with each other or are mixed with a  
corresponding component, a hydrogel material will form. One may react a PEG  
35 component with a non-PEG component, controlling the molecular weight or  
20 hydrophilicity of either component to manipulate the mechanical characteristics, the  
permeability, and the water content of the resulting biomaterial. These materials are  
generally useful in medical implants, as described in more detail below.

40 In the formation of biomaterials, especially biomaterials that are desired to degrade  
*in vivo*, peptides provide a very convenient building block. It is straightforward to  
45 25 synthesize peptides that contain two or more cysteine residues, and this component can  
then readily serve as the nucleophilic precursor component of a biomaterial, especially a

5 hydrogel biomaterial. For example, a peptide with two free cysteine residues will readily  
form a hydrogel when mixed with a PEG triacrylate at physiological or slightly higher pH  
10 (e.g., 8 to 9; the gelation will also proceed well at even higher pH, but at the potential  
expense of self-selectivity). When the two liquid precursor components are mixed  
5 together, they react over a period of a few minutes to form an elastic gel, consisting of a  
network of PEG chains, bearing the nodes of the network, with the peptides as connecting  
15 links. The peptides can be selected as protease substrates, so as to make the network  
capable of being infiltrated and degraded by cells, much as they would do in a protein-  
based network. The gelation is self-selective, meaning the peptide reacts mostly with the  
20 10 PEG component and no other components, and the PEG component reacts mostly with  
the peptide and no other components; if desired, one can design and incorporate  
biofunctional agents to provide chemical bonding to other species (e.g., a tissue surface).  
25 These gels are operationally simple to form: one mixes two liquid precursors, one  
containing the peptide and the other containing the functionalized PEG. Because, in this  
15 example, physiological saline can serve as the solvent, and because minimal heat is  
generated by reaction, and because neither the PEG triacrylate nor the peptide can readily  
30 diffuse inside cells, the gelation can be carried out *in vivo* or *in vitro*, in direct contact  
with tissue, without untoward toxicity. It is clear that polymers other than PEG may be  
35 used, either telechelically modified or modified on their side groups.

#### 20 *Protease sites*

40 One special feature of the chemical cross-linking scheme of this invention is that it  
is self-selective, meaning that it does not react with other features on peptides or proteins.  
Thus, one can employ peptides as one component, as described above, and not chemically  
45 25 react with side groups on the peptide other than cysteine residues. This means that a  
variety of bioactive peptides can be incorporated into the resulting biomaterial structure.

5 For example, a peptide used as a dithiol for cross-linking purposes can be designed to be  
a substrate for an enzyme used by cells migration through tissues and remodel tissues  
10 (e.g., as a substrate for plasmin, elastase or matrix metalloproteinases (MMPs), such as  
collagenase). The degradation characteristics of the gels can be manipulated by changing  
5 the details of the peptide that serves as the cross-linking nodes. One may make a gel that  
is degradable by collagenase, but not plasmin, or by plasmin, but not collagenase.  
15 Furthermore, it is possible to make the gel degrade faster or slower in response to such an  
enzyme, simply by changing the amino acid sequence so as to alter the  $K_m$  or  $k_{cat}$ , or both,  
of the enzymatic reaction. One can thus make a biomaterial that is biomimetic, in that it  
20 10 is capable of being remodeled by the normal remodeling characteristics of cells.

#### *Adhesion sites*

25 One can incorporate peptide sites for cell adhesion, namely peptides that bind to  
adhesion-promoting receptors on the surfaces of cells into the biomaterials of the present  
invention. It is straightforward to incorporate a variety of such adhesion-promoting  
30 15 peptides, such as the RGD sequence from fibronectin or the YIGSR sequence from  
laminin. As above, this can be done, for example, simply by mixing a cysteine-  
containing peptide with PEG diacrylate or triacrylate, PEG diacrylamide or triacrylamide  
35 or PEG diquinone or triquinone a few minutes before mixing with the remainder of the  
thiol-containing precursor component. During this first step, the adhesion-promoting  
20 peptide will become incorporated into one end of the PEG multiply functionalized with a  
conjugated unsaturation; when the remaining multithiol is added to the system, a cross-  
40 linked network will form. Thus, for example, when an adhesion peptide containing one  
cysteine is mixed with a PEG triacrylate (at, e.g., 0.1 mole of peptide per mole of acrylate  
end group), and then a protease substrate peptide containing two cysteine residues is  
45 25 added to form the three-dimensional network (at, e.g., equimolar less 0.1 mole peptide per



5 mole of acrylate end group), the resulting material will be highly biomimetic: the  
combination of incorporated adhesion sites and protease sites permits a cell to establish  
10 traction in the material as it degrades a pathway for its migration, exactly as the cell  
would naturally do in the extracellular matrix *in vivo*. In this case, the adhesion site is  
5 pendantly incorporated into the material. One could also incorporate the adhesion site  
directly in to the backbone of the material. This could be done in more than one way.  
15 One way would be to include two or more thiols (e.g., cysteine) in the adhesion peptide or  
protein. One could alternatively synthesize the adhesion peptide (e.g., using solution  
phase chemistry) directly onto a polymer, such as PEG, and include at least one thiol  
20 0 (e.g., cysteine) or conjugated unsaturation per chain end.

#### *Growth factor binding sites*

25 One can further enhance the biomimetic nature of the biomaterials of the present  
invention, especially when they are formed from water-soluble components so as to be  
hydrogels, by the incorporation of growth factor binding domains. For example, heparin-  
30 15 binding peptides can be employed to bind heparin, which can in turn be employed to bind  
heparin-binding growth factors, such as bFGF, VEGF, BMP or TGF $\beta$ . As such, if the  
heparin-binding growth factor, heparin, and the activated heparin-binding peptide were  
35 mixed with the activated PEG (similarly as described in the preceding section), the  
resulting gel will slowly release the growth factor, holding most of it until an invading  
20 cell released the growth factor by degradation of the gel. This is one of the natural  
40 functions of the extracellular matrix *in vivo*, to serve as a depot for growth factors which  
become released in injury by local cellular activity. Another related way to sequester  
heparin-binding growth factors would be more directly through the use of covalently  
45 incorporated heparin mimics, for example, peptides with negatively charged side chains,  
25 that directly bind growth factors. Moreover, since the biomaterial itself is a network, it

5 can be used to release a growth factor that is simply physically incorporated and is  
released slowly by degradation or diffusion, or a combination thereof. It should be  
understood that because the gelation chemistry is self-selective, the growth factor itself  
10 and the other bioactive peptides are not chemically modified so as to destroy their  
biological activity. This important aspect of self-selectivity obviates the need, for  
5 example, to encapsulate the growth factor in polymer particles (to thereby protect it from  
the gelation chemistry, if the gelation chemistry were to react with side groups that are  
15 present free on the growth factor, such as the epsilon amines present on the side chains of  
lysine in the protein).

#### 10 **Drug Delivery from Hydrogels Formed by Conjugate Addition Reactions**

Hydrogels are particularly useful for the delivery of protein therapeutics.  
25 Hydrogels are biocompatible, and provide a gentle environment for proteins so as to  
minimize denaturation of the proteins. Conjugate addition reactions with thiols are  
utilized for the production of gels in the presence of proteins, because of the self-  
30 15 selectivity of these reactions as compared with nucleophilic substitution reactions, free-  
radical reactions or reactions involving amines for reactivity. Thus, the proteins are  
physically entrapped within the gels. Additionally, degradable segments can be  
35 incorporated within the polymers that form the hydrogel, and via degradation of segments  
within the gel, the proteins will be released as the gel degrades. A particularly useful  
20 embodiment of the invention occurs in the case when the conjugate addition reaction  
itself leads to a structure that is particularly prone to hydrolysis.

40 In the majority of cases, protein drugs or high molecular weight therapeutics such  
as antisense oligonucleotides or genes are delivered from degradable hydrophobic  
materials, such as polylactic acid. However, we describe more hydrophilic materials,  
45 25 such as cross-linked polyethylene glycol functionalized with thiols, with conjugated

5 unsaturations, or both. Other examples exist, including photo-cross-linked polyethylene glycol (Pathak et al., Journal of the American Chemical Society 114:8311-8312, 1992) and polyethylene glycol cross-linked by nucleophilic substitution reactions (Zhao et al.,  
10 Polymer Preprints 38:526-527, 1997; WO 99/2270; WO 99/34833, and WO 99/14259).

5 The cross-linking via conjugate addition chemistries with thiols exhibits excellent self-selectivity, in that reaction between the conjugated group and other groups, such as amines, in proteins, will be quite slow. When the protein to be incorporated contains a free thiol, this will be reacted with the biomaterial system unless it is otherwise protected or reacted.

10 An additional advantage to the use of biomaterials formed by conjugate addition with thiols to encapsulate and release proteins arises due to the chemistry of groups generated by the conjugate addition cross-linking. If the conjugated group is an acrylate, then a relatively unstable ester is present in the system. If the acrylate were subjected to  
25 free-radical cross-linking, it has been found that such gels degrade only very slowly at pH 7.4 and 37°C, with a gel that degrades over the period of about a year. However, if the acrylate group is reacted with a thiol, the ester of the acrylate group hydrolyzes with a half-life of approximately 3 weeks, producing gels that degrade over about 3 weeks (as described below). Whereas in the case of free-radical cross-linking, special groups must  
35 be included between the polyethylene glycol and the acrylate to promote degradation of the gel (such as polylactic acid oligomers; Pathak, *supra*), no special groups are required between the acrylate and the polyethylene glycol in the case of the conjugate addition cross-linking. One can employ more stable linkers between the conjugated unsaturation and the polymer, and then incorporated a domain that is degradable by hydrolysis, such as  
40 an oligomer of glycolic acid, lactic acid, epsilon caprolactone, or trimethylene carbonate, between the polymer and the conjugated unsaturation, to obtain degradation of the biomaterial by degradation of these domains.

## Biomedical Applications for Hydrogels

Hydrogels are polymeric materials that are highly swollen with water. For many applications, hydrogels are especially useful. Hydrogels are of interest for myriad biomedical applications. These include but are not limited to barrier applications (adhesion preventives, sealants), drug delivery devices, tissue engineering and wound healing scaffolds, materials for cell encapsulation and transplantation, materials for surgical augmentation of tissues and materials for sealants and adhesives. An incomplete but illustrative list of applications for hydrogels in biomedicine follows:

1. Hydrogels for adhesion prevention are desirable to minimize unwanted post-operative or other post-traumatic adhesions. Such adhesions can be proteinaceous or cellular, or both. For example, postoperative abdominopelvic adhesions can lead to chronic pain, bowel obstruction, and infertility. As a second example, unwanted adhesion between blood platelets and the blood vessel wall surface after balloon angioplasty in the vascular system can lead to thrombosis and restenosis. Materials cured *in situ* upon a surgical site may be useful in preventing postoperative adhesions, especially when these materials degrade over a period of several days to weeks. Materials cured *in situ* upon the surface of an injured artery may be useful in preventing thrombosis upon the site of vascular trauma associated with catheter intervention, deployment of a stent, or surgery.

2. Hydrogels as glues or sealants are desirable to seal leaks in tissues that isolate (gas or liquid phase) fluid-containing cavities. Some examples are blood vessels, the skin, the lung, the dura barrier, and the intestine. The materials may be useful internally, for example, in sealing air leaks on the lung, and externally, for example, in closing incisions on the skin.

3. Hydrogels can also be useful as localized drug delivery devices. A drug may be any biologically active molecule, for example, a natural product, synthetic drug, protein (such as growth factors or enzymes), or genetic material. The functional properties of

5 such a drug must be preserved by its carrier. The drug may be released by diffusive  
mechanisms or by degradation of the gel carrier through a variety of mechanisms (such as  
10 hydrolysis or enzymatic degradation) or by other sensing mechanisms (for example, pH  
induced swelling). Given that many drugs contain reactive groups, it is important that the  
5 material that serves as the carrier not react with the material in an undesirable manner; as  
such, the high self-selectivity of reactions between conjugated unsaturations and thiols is  
15 very useful in drug encapsulation.

4. Hydrogels as scaffolds are desirable for tissue engineering and wound healing  
applications: nerve regeneration, angiogenesis, and skin, bone and cartilage repair and  
20 regeneration. Such scaffolds may be introduced to the body pre-seeded with cells or may  
depend upon cell infiltration from outside the material in the tissues near the implanted  
biomaterial. Such scaffolds may contain (through covalent or non-covalent bonds) cell  
25 interactive molecules like adhesion peptides and growth factors.

5. Hydrogels also have biomedical applications as cell transplant devices. Such  
15 devices serve to isolate cells (e.g., allograft or xenograft) from a host's defense system  
(immunoprotect) while allowing selective transport of molecules such as oxygen, carbon  
30 dioxide, glucose, hormones, and insulin and other growth factors, thus enabling  
encapsulated cells to retain their normal functions and to provide desired benefits, such as  
35 the release of a therapeutic protein that can diffuse through the immunoprotection  
hydrogel membrane to the recipient.  
20

6. Hydrogels can be responsive to their environment. They can be designed to  
40 increase network formation, and thus attachment, between gel and tissue because when  
initially injected the components are water borne and water soluble. Upon transition of  
the active stimuli (e.g., temperature or pH) one or both of the precursors become water  
25 insoluble giving lower average water content and result in increased stiffness and  
45 improved mechanical properties of the resulting gel.

5 In some of these examples cited above, it is desirable to form therapeutic  
hydrogels at their final destination in the body. Implantable materials which can be  
10 injected in the liquid phase to a target site where they can then be transformed into solid  
materials are therefore of interest. The shape of such an implant can match the tissue  
5 topography, and a relatively large implant can be delivered through minimally invasive  
methods. Often, good adhesion to the underlying tissue substrate can be achieved, for  
15 example, by intimate penetration of the liquid precursors into texture on the tissue surface  
or by phase interpenetration to form an interpenetrating polymer network between the  
biomaterial polymer network and the natural tissue extracellular materials, which are also  
20 a polymer network. One can also design additional materials to serve a role as coupling  
agent to enhance adhesion. For example, one can design a heterobifunctional coupling  
agent, with an activated ester (such as an N-succinimidyl activated ester derivative) or an  
25 epoxide group on one end and a conjugated structure that reacts slowly with amines on  
the other end. Such an agent would react with proteins on the tissue surface when applied  
15 to the tissue surface and would then immobilize conjugated groups for chemical  
incorporation into the biomaterial network during polymerization or cross-linking. This  
30 pre-treatment step would thereby introduce upon the surface of the tissue chemical groups  
that could participate in the self-selective cross-linking between the two components of  
the final precursor solution.

35 20 There are many ways to form biomaterials including hydrogels. However,  
materials made in contact with sensitive biological materials, including cells or tissue, or  
intended for implantation or other contact with the body are subject to special constraints.  
40 In the text below, the situation of formation of a biomaterial hydrogel is considered,  
because of the special usefulness of biomaterial hydrogels. The approaches are generally  
25 the same for non-hydrogel materials, and the approaches described below should be  
45 understood to be generalizable. The network formation process must proceed in

5 relatively mild conditions with regard to solvent system, temperature and exothermicity,  
and pH. Precursors and products (of gelation reactions and of gel degradation) should be  
10 substantially non-toxic, with toxic being defined as inducing a medically unacceptable  
tissue reaction in a medically relevant context.

5 The approaches described herein using conjugate addition reactions with thiols to  
form biomaterials simplify the process of gel formation (no light or temperature changes  
15 are required) and add greatly to usefulness by being self selective (in general not reacting  
with proteins that are incorporated as biopharmaceuticals or are present on cell and tissue  
surfaces). Furthermore, because of the self-selectivity, it is possible to much more  
20 0 flexibly incorporate peptides into the biomaterial itself, for example, as protease cleavage  
sites (to provide degradation), cell adhesion sites, or heparin or growth factor binding  
sites.

25 There exist numerous applications in medicine where *in situ* cross-linking is  
desired but where hydrogels are not desired. These can include applications where a high  
15 strength material is desired. High strength hydrogels can be formed, but in general non-  
hydrogel materials can be stronger. These materials can be obtained either by cross-  
30 linking, using the scheme of this invention, in the presence of a low toxicity non-aqueous  
solvent, such as ethylacetate, a low molecular weight PEG, or from cross-linking neat  
without any solvent, from liquid precursors. For example, a hydrolytically degradable  
35 20 strong material could be formed from a low molecular weight poly(epsilon caprolactone)  
diacrylate (which is a liquid) as a hydrophobic component. Such materials can be either  
40 linear polymeric biomaterials or cross-linked polymeric biomaterials. This may also be  
achieved by using precursors that exhibit sensitivity to pH, temperature or other stimuli  
which can be manipulated. In this manner, the precursors will undergo a transition from  
45 25 soluble to insoluble after/during application. This will allow easy handling but allow the  
improvement of mechanical properties by using non-hydrogel (low water content)

5 materials.

It is possible to prepare structural materials with significant mechanical strength *in situ* using conjugate addition with thiols. If high cross-linking density and/or low water  
10 content are used, gels or materials with high mechanical strength can be obtained.

5 Multifunctional, low molecular weight precursors with limited or no water solubility can be combined to form strong cross-linked materials. These insoluble or partially soluble precursors can be combined, if they are liquid, by dispersing in aqueous with or without  
15 the assistance of emulsifiers. This emulsifier may be nontoxic or minimally toxic surfactants, such as sorbitan monooleate, or it may be a protein such as albumin.

20 Inorganic particles can also assist in the water dispersion of such precursors. The mechanical properties of the structural gels obtained by this method can be modified by the addition of inorganic particles, hydrophilic or hydrophobic additives, or by the use of  
25 multimodal molecular weight precursors (precursors with multiple discrete molecular weights). The addition of inorganic particles increases the stiffness of the cross-linked material and can increase the ultimate strength and the fatigue resistance of the material.  
15 The addition of hydrophilic additives can be used to increase the water content and to soften the materials. Depending on the chemical composition, the addition of  
30 hydrophobic additives can be used to reduce the water content of the gel and can be used to harden and/or strengthen the materials. This may also be used to enhance elasticity.  
35 The density of cross-linking can be affected by the molecular weight of the original precursors. Increase of the molecular weight can reduce the cross-linking density and be  
20 used to modulate the mechanical properties of the final biomaterial.

## 40 II. Cross-linking chemistry

As used herein, the symbol **P** is employed to indicate the part of a molecule that  
45 25 lies between two reactive sites (telechelic sense) or is grafted with reactive sites (grafted



5 sense). With telechelic polymers, P will lie between two strong nucleophiles, such as two  
thiols, or between two conjugated unsaturations (e.g., in the case of a PEG diacrylate or a  
10 PEG dithiol, P is a PEG chain). In the case of a PEG triquinone or trithiol, P is a three-  
armed, branched PEG. In the case of a block copolymeric acrylate-(lactic acid oligomer)-  
5 PEG-(lactic acid oligomer)-acrylate or quinone-(lactic acid oligomer)-PEG-(lactic acid  
oligomer)-quinone, P is the (lactic acid oligomer)-PEG-(lactic acid oligomer) block  
15 copolymer. In the case of a graft copolymer (e.g., polylysine-graft-(PEG acrylate) or  
polylysine-graft-(PEG quinone) or polylysine-graft-(PEG thiol)), in which the geometry  
of the polymer is as a bottle-brush with the tips of the bristles containing either the  
20 conjugated unsaturations or the strong nucleophile, P is polylysine-graft-(PEG). P can  
also present the reactive groups in the side chains: every polymer bearing alcohols or  
amines in the side chains is easily acrylated, for example, in order to present multiple  
25 conjugated unsaturated groups for the conjugate addition reaction. Polymers containing  
carboxylic acids can be derivatized to expose, for example, quinines groups. P need not  
15 be polymeric in the usual sense of the word. For example, in the case of ethylene glycol  
diacrylate or diquinone, P is the ethylene unit. In the case of a peptide, for example,  
30 YCXXXXXXXXCY (SEQ ID NO: 1) or CXXXXXXXXC (SEQ ID NO: 2), where C is the  
amino acid cysteine and X and Y are other amino acids, such that XXXXXXXX (SEQ ID  
35 NO: 3) could be a sequence that functions as a substrate for a protease such as  
20 collagenase, P is XXXXXXXX. The length of XXXXXXXX or the number of X (e.g., X<sub>n</sub>) can  
be any length or number (n=0). In the case of 1,2 ethylene dithiol, P is the ethylene.  
40 Thus, P is the molecular part of the precursor component that is interposed between the  
two, or more, reactive groups on the precursor component. It is often convenient when  
this is polymeric or oligomeric, but neither case is necessary; small molecules are also of  
25 interest and use. Examples of small molecules which may be used include, but are not  
45 limited to reduced sugars or analogous compounds, such as mannitol, erythritol,

5 pentaeritrol, trimethylol propane, and glycerol, which can be totally or partially acrylated,  
or reacted with beta-mercapto propionic acid to give thiols. Di- or multicarboxylic acids,  
10 such as EDTA, citric acid, succinic acid, and sebacic acid, can be converted to quinones.

#### Definition of Michael-type reaction

5 The 1,4 addition reaction of a nucleophile on a conjugate unsaturated system is  
15 referred to as a Michael-type reaction. The addition mechanism could be purely polar, or  
proceed through a radical-like intermediate state(s); Lewis acids or appropriately  
designed hydrogen bonding species can act as catalysts. The term conjugation can refer  
20 both to alternation of carbon-carbon, carbon-heteroatom or heteroatom-heteroatom  
multiple bonds with single bonds, or to the linking of a functional group to a  
0 macromolecule, such as a synthetic polymer or a protein. Double bonds spaced by a CH  
or CH<sub>2</sub> unit are referred to as homoconjugated double bonds.  
25

Michael-type addition to conjugated unsaturated groups can take place in good to  
quantitative yields at room or body temperature and in mild conditions with a wide  
30 15 variety of nucleophiles (Pathak, *supra*; Mathur et al., Journal of Macromolecular Science-  
Reviews In Macromolecular Chemistry and Physics," C36:405-430,1996; Moghaddam et  
al., Journal of Polymer Science: Part A: Polymer Chemistry 31:1589-1597, 1993; and  
35 Zhao, *supra*). Conjugated unsaturated groups, such as vinyl sulfones (Pathak, *supra*) or  
acrylamides (Mathur, *supra*), have been used to link PEG or polysaccharides to proteins  
20 through Michael-type reactions with amino- or mercapto-groups.

40 The innovation of the present invention consists in the fact that a biocompatible  
gelling of biomaterial precursors to form a biomaterial is rapidly provided by the use of a  
wide variety of conjugated unsaturated compounds reacting with thiols in a self-selective  
45 25 manner. The gel formation kinetics and the mechanical and transport properties of the  
product are tailored to the needs of the application. The possibility to incorporate

proteinaceous or peptidyl material is envisaged mainly in order to obtain a proteolytically degradable material or for specific recognition processes within it, but primarily by reaction with intentionally incorporated cysteine residues; pure protein PEGylation is outside of the scope of the present invention, since it does not result in a biomaterial. Groups such as maleimides and vinylsulfones are useful in these cross-linking reactions, but these tend to be less useful than others because of a relatively high rate of reactivity with amines relative to other nucleophiles such as compared to some of the conjugated systems described below. As such, the use of conjugated unsaturations that display lower overall reactivity, including quinones and acrylates.

#### Conjugated unsaturated structures

It is possible to perform Michael-type addition reactions on a wide variety of conjugated unsaturated compounds. In the structures shown below, an oligomeric or polymeric structure is indicated as P. Various possibilities for the specific identity of P are discussed further herein. P can be coupled to reactive conjugated unsaturated groups in structures such as those numbered 1 to 20.

In the drawings, P is intended as terminated with a CH<sub>2</sub>, CH or C group.

Reactive double bonds can be conjugated to one or more carbonyl groups in a linear ketone, ester or amide structure (1, 2) or to two in a ring system, as in a maleic or paraquinoid derivative (3, 4, 5, 6, 7, 8, 9, 10). In the latter case the ring can be fused to give a naphthoquinone (6, 7, 10) or a 4,7-benzimidazoledione (8) (Pathak, *supra*) and the carbonyl groups can be converted to an oxime (9, 10). The double bond can be conjugated to a heteroatom-heteroatom double bond, such as a sulfone (11), a sulfoxide (12), a sulfonate or a sulfonamide (13), a phosphonate or phosphonamide (14). Finally, the double bond can be conjugated to an electron-poor aromatic system, such as a 4-vinylpyridinium ion (15). Triple bonds can be used in conjugation with carbonyl or

5 heteroatom-based multiple bonds (16, 17, 18, 19, 20).

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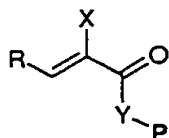
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## Chemical Structures:

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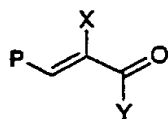
X = H, CH<sub>3</sub>      R = H      Y = NH, O, 1,4-Ph  
 CN, COOW      R = H, W, Ph      Y = NH, O, 1,4-Ph

W = C1-C5 linear aliphatic chain

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1

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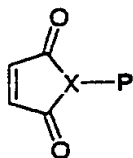
X = CN, COOW      Y = OW, Ph

W = C1-C5 linear aliphatic chain

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2

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X = N, CH

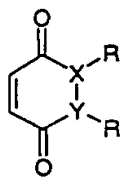
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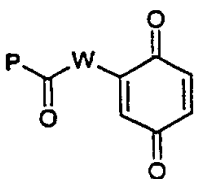


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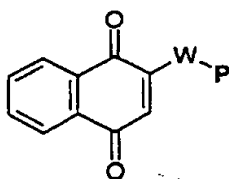
**A** X = CH Y = CH R = H, W-P (W = NH, O, nihil)

**B** X = N Y = N R = H, P

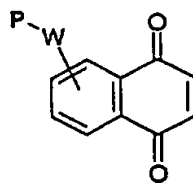
**C** X-Y = C=C R = W-P (W = NH, O, nihil)



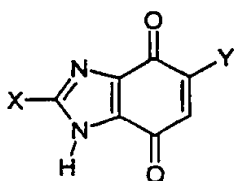
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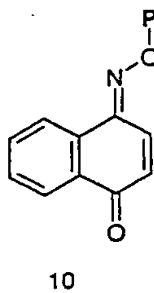
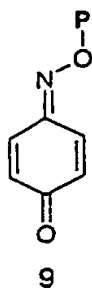
X, Y = H, P  
P, P  
P, H  
P, aliphatic chain

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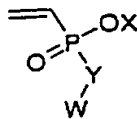
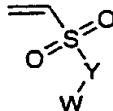
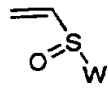
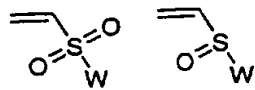
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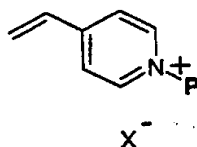
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Y = O, NH  
X = alkali or alkali  
earth metal ion, P  
W = P, 1,4-Ph-P

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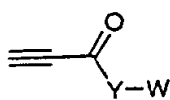
X = halogen, sulphonate

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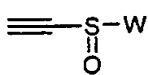
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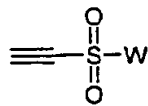
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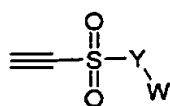
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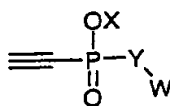
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Y = O, NH  
X = alkali or alkali  
earth metal ion, P  
W = P, 1,4-Ph-



5 Structures such as 1 and 2 are based on the conjugation of a carbon-carbon double  
bond with one or two electron-withdrawing groups. One of them is always a carbonyl,  
increasing the reactivity passing from an amide, to an ester, and then to a phenone  
10 structure. The nucleophilic addition is easier upon decreasing the steric hindrance, or  
5 increasing the electron-withdrawing power in the alpha-position:  $\text{CH}_3 < \text{H} < \text{COOW} < \text{CN}$ .

The higher reactivity obtained by using the last two structures can be modulated by  
15 varying the bulkiness of the substituents in the beta-position, where the nucleophilic  
attack takes place; the reactivity decreases in the order  $\text{P} < \text{W} < \text{Ph} < \text{H}$ . So, the position of P  
too can be used to tune the reactivity towards nucleophiles. This family includes some  
20 10 compounds for which a great deal is known about their toxicology and use in medicine.  
For example, water-soluble polymers with acrylates and methacrylates on their termini  
are polymerized (by free radical mechanisms) *in vivo*, in hydrogel sealants and bone  
25 cements, respectively. Thus, acrylate and methacrylate-containing polymers have been  
seen in the body before in clinical products, but for use with a dramatically different  
15 chemical reaction scheme.

30 The structures 3-10 exhibit very high reactivity towards nucleophiles, due both to  
the *cis* configuration of the double bond and the presence of two electron-withdrawing  
groups.

35 20 Unsaturated ketones react faster than amides or imides, due to the stronger  
electronegativity of these carbonyl groups. So, cyclopentendione derivatives react faster  
than maleimidic ones (3), and para-quinones react faster than maleic hydrazides (4) and  
also faster than cyclohexanones, due to more extended conjugation. The highest reactivity  
40 is shown by naphthoquinones (7).

25 P can be placed in positions where it does not reduce the reactivity of the  
unsaturated group, that is in the opposite part of the ring (3, 5), on another ring (7, 8) or  
45 O-linked through a para-quinone mono-oxime (9, 10). P can be also linked to the

reactive double bond (6, 8), if the nucleophilic addition rate is to be decreased.

The activation of double bonds to nucleophilic addition can be obtained also by using heteroatoms-based electron-withdrawing groups. In fact, heteroatom-containing analogous of ketones (11, 12), esters and amides (13, 14) provide a similar electronic behavior. Structures 13 and 14 can also be used as easily hydrolyzable groups, that can promote a quick gel degradation. The reactivity towards nucleophilic addition increases with electronegativity of the group, that is in the order  $11 > 12 > 13 > 14$ , and is enhanced by the linkage with an aromatic ring. A strong activation of double bonds can also be obtained, using electron-withdrawing groups based on aromatic rings. Any aromatic structure containing a pyridinium-like cation (e.g., derivatives of quinoline, imidazole, pyrazine, pyrimidine, pyridazine, and similar  $sp^2$ -nitrogen containing compounds) strongly polarizes the double bond and makes possible quick Michael-type additions.

Carbon-carbon triple bonds, conjugated with carbon- or heteroatom-based electron-withdrawing groups, can easily react with sulphur nucleophiles, to give products from simple and double addition. The reactivity is influenced by the substituents, as for the double bond-containing analogous compounds.

The formation of ordered aggregates (liposomes, micelles) or the simple phase separation in water environment increase the local concentration of unsaturated groups and so the reaction rate. In this case, the latter depends also on the partition coefficient of the nucleophiles, which increases for molecules with enhanced lipophilic character.

#### Nucleophiles

The nucleophiles that are useful are those that are reactive towards conjugated unsaturated groups via addition reactions. The reactivity of the nucleophile depends on the identity of the unsaturated group, as discussed in more detail elsewhere herein, but the identity of the unsaturated group is first limited by its reaction with water at physiologic

5 pH. Thus, the useful nucleophiles will generally be more nucleophilic than H<sub>2</sub>O at  
physiologic pH. Preferred nucleophiles will be ones that are commonly found in  
biological systems. for reasons of toxicology, but ones that are not commonly found free  
10 in biological systems outside of cells. Thus, while there may be examples in which  
amines can be employed as effective nucleophiles, the most preferred nucleophile is the  
thiol.

15 Thiols are present in biological systems outside of cells in paired form, as disulfide  
linkages. When the highest degree of self-selectivity is desired (e.g., when a therapeutic  
protein is incorporated, when the gelation reaction is conducted in the presence of tissue  
20 and chemical modification of that tissue is not desirable), then a thiol will represent the  
strong nucleophile of choice.

25 There are other situations, however, when the highest level of self-selectivity may  
not be necessary. This would include situations when no therapeutic protein is  
incorporated and when the gelation reaction is conducted *in situ*, but when chemical  
15 bonding to the tissue is either desirable or is not undesirable. In these cases, an amine  
may serve as an adequate nucleophile. Here, particular attention is paid to the pH, in that  
the deprotonated amine is a much stronger nucleophile than the protonated amine. Thus,  
30 for example, the alpha amine on a typical amino acid (pK as low as 8.8 for asparagine,  
average of 9.0 for all 20 common amino acids except proline) has a much lower pK than  
35 the side chain epsilon amine of lysine (pK 10.80). As such, if particular attention is paid  
20 to the pK of an amine used as the strong nucleophile, substantial self-selectivity can be  
obtained. Proteins have only one alpha amine (on the N-terminus). By selection of an  
40 amine with a low pK, and then formulation of the final precursor solution such that the  
pH were near that pK, one could favor reaction of the unsaturation provided with the  
25 amine provided, rather than other amines present in the system. In cases where no self-  
45 selectivity is desired, one need pay less attention to the pK of the amine used as the

5 nucleophile, however to obtain reaction rates that are acceptably fast one must adjust the  
pH of the final precursor solution such that an adequate number of these amines are  
deprotonated.

10 In summary, thiols are the preferred strong nucleophile of this invention, for  
5 reasons of pH in the precursor solution and maximal self-selectivity, but there are  
situations in which amines will also serve as useful strong nucleophiles; the usefulness of  
particular nucleophiles depends upon the situation envisioned and the amount of self-  
15 selectivity desired.

20 The concept of nucleophilic group is extended herein, so that the term is  
10 sometimes used to include not only the functional groups themselves (e.g., thiol or  
amine), but also molecules which contain the functional group (e.g., cysteine or cystyl  
residue, or lysine or lysyl residue).

25 The nucleophilic groups may be contained in molecules with great flexibility in  
overall structure. For example, a difunctional nucleophile could be presented in the form  
15 of Nuc-P-Nuc, where P is used in the sense described herein and Nuc refers to the  
nucleophile. Likewise, a branched polymer P could be derivatized with a number of  
30 nucleophiles to create P-(Nuc)<sub>i</sub>, where i=2 would be useful. Nuc needs not be displayed  
at the chain termini of P, for example, a repeating structure could be envisioned: (P-  
Nuc)<sub>i</sub>, where i=2 would be useful. Clearly, not all of the P or the Nuc in such a structure  
35 need to be identical. It is only necessary that one nucleophilic precursor contain greater  
20 than or equal to two such Nuc groups.

40 Likewise, similar structures of P and the conjugated unsaturated groups described  
in detail above may be formed. It is only necessary that one conjugated unsaturated  
precursor contain greater than or equal to two such conjugated unsaturated groups.

25 It should be noted and understood, that it is not necessary that both precursor  
45 components, for example, both the nucleophilic precursor component and the conjugated

5 unsaturated precursor component, actually be polymeric in the usual sense of the word. It  
is only the functionality that matters. In practice, it is convenient if at least one  
component is polymeric in the usual sense of the word, but this is not absolutely  
10 necessary. For example, useful materials result from the reaction of a PEG triacrylate  
with dicysteine, and likewise, useful materials result from the reaction of a PEG trithiol  
and a low molecular weight diacrylate. Finally, useful materials for some applications  
15 also result from reaction of a dicysteine and a low molecular diacrylate.

In practice, it is convenient and useful when one or more precursor component is  
polymeric in the usual sense of the word. In these cases, P can be synthetic hydrophilic  
20 10 polymers, synthetic hydrophobic polymeric liquids, synthetic hydrophobic polymers that  
are soluble in solvents of acceptable toxicity or biological influence for the envisioned  
application, biosynthetic proteins or peptides, naturally occurring proteins or processed  
25 naturally occurring proteins, or polysaccharides.

#### Hydrophilic polymers

30 15 In preferred embodiments, the synthetic polymer P can be poly(ethylene glycol),  
poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(acrylic  
acid), poly(ethylene-co-acrylic acid), poly(ethyloxazoline), poly(vinyl pyrrolidone),  
poly(ethylene-co-vinyl pyrrolidone), poly(maleic acid), poly(ethylene-co-maleic acid),  
35 poly(acrylamide), or poly(ethylene oxide)-co-poly(propylene oxide) block copolymers  
20 This is not an exhaustive list as other hydrophilic polymers could also be used.

P can also be copolymers, block copolymers, graft copolymers, or random  
40 copolymers. Blocks, which are polymerized on the ends of the hydrophilic polymers, can  
be composed of, for example, lactic acid, glycolic acid, epsilon-caprolactone, lactic-co-  
glycolic acid oligomers, trimethylene carbonate, anhydrides, and amino acids, for  
45 25 example, to confer degradability by hydrolytic or enzymatic means. This list is not

5 exhaustive; other oligomers may also be used for block copolymers.

Random copolymers can be based on vinyl alcohol, such as poly(N-vinylpyrrolidone-co-vinyl alcohol) or poly(ethylene-co-vinyl alcohol), with different  
10 compositions, can be derivatized with conjugated unsaturated groups, such as acrylates, benzoquinones, naphthoquinones and others. The vinyl alcohol copolymers can be  
5 functionalized with  $(CH_2)_n$  COOH groups by reaction with  $\omega$ -bromo-carboxylic acids. The resulting polymers or acrylic or methacrylic acid copolymers can be used for the  
15 attachment of quinone groups. Comonomer composition and extent of functionalization do not influence dramatically the reaction rates, unless they determine solubility or phase  
20 transition. On the other hand, they determine the final mechanical properties.

10 It should be noted that one component P could even be a solid, such as a colloidal particle with either nucleophiles or sites of conjugated unsaturation upon it.

#### 25 **Proteins and biosynthetic proteins**

P may be a protein. The protein can be a naturally occurring or recombinant  
15 protein. In general terms, the recombinant proteins are any length amino acid material generated through recombinant DNA technology. Examples of components these can  
30 have include peptide sequences which encode degradation sites for proteases, peptide sequences for other biological signals and non biointeractive sequences.

35 Any naturally occurring protein can also be P. More specifically, a naturally occurring protein is composed of several Ps which are separated by nucleophiles. For  
20 example, serum albumin, a 584 amino acid protein, contains 5.7 % cysteine, 9.9 % lysine and 3.1 % tyrosine. The amino acid sequences which occur between, for example,  
40 cysteine, tyrosine and lysine make up distinct Ps. While albumin in its natural state may be less than useful for the purposes of cross-linking by conjugate addition reactions  
45 between conjugated unsaturations and thiols on the protein, albumin can be readily  
25

5 processed by reduction so as to form a poly(amino acid) with some or all of its cysteine residues free or it can be chemically derivatized to introduce multiple thiol groups.

## 10 Peptides

In some instances, P may be a peptide or a polypeptide, where the nucleophile is  
5 the amino acid cysteine, resulting in peptides of structures similar to H<sub>2</sub>N-  
15 CXXXXXXCXXXXXC-COOH (SEQ ID NO: 4) or H<sub>2</sub>N-CXXXXXXC-COOH (SEQ ID  
NO: 5), where C is the one-letter representation of cysteine, and X represents any amino  
acid except cysteine, in one embodiment, or Acetyl-NH-YXXXXXXYXXXXXY-COOH  
20 (SEQ ID NO: 6) where Y is the one-letter representation of tyrosine, and X represents any  
10 amino acid except cysteine or tyrosine, in another embodiment. The length of XXXXXX  
(SEQ ID NO: 7) or the number of X (e.g., X<sub>n</sub>) can be any length or number (n=0). It is  
25 particularly useful when the sequences in the domains shown as XXXXXX above are  
substrates for enzymes that are involved in cell migration (e.g., as substrates for enzymes  
such as collagenase, plasmin or elastase), although the domains need not be limited to  
30 these. One such particularly useful sequence, as a substrate for the enzyme plasmin, is  
described in the examples. A variety of such peptides may be learned from a study of the  
literature of these enzymes. For example, such a study shows substrate sites for the  
35 important protease plasmin (Table 1; SEQ ID NOS: 8-24):

Table 1. Plasmin Substrate Sites found in Fibrin (ogen) (Fg)\*\*

| Arginyl Sites |    |    |     |     |     |                   | Reference |
|---------------|----|----|-----|-----|-----|-------------------|-----------|
| P3            | P2 | P1 | P1' | P2' | P3' | Fg chain and site |           |
| G             | P  | R+ | V*  | V*  | E-  | $\alpha$ 19       | 3         |
| N             | N  | R+ | D-  | N   | T   | $\alpha$ 104      | 2, 4      |
| Y             | N  | R+ | V*  | S   | E-  | $\alpha$ 110      | 2         |
| Q             | M* | R+ | M*  | E-  | L*  | $\alpha$ 239      | 1         |
| G             | F* | R+ | H+  | R+  | H+  | $\alpha$ 491      | 5         |
| G             | Y  | R+ | A*  | R+  | P   | $\beta$ 42        | 2, 3      |
| Lysyl Sites   |    |    |     |     |     |                   | Reference |
| P3            | P2 | P1 | P1' | P2' | P3' | Fg chain and site |           |
| Y             | Q  | K+ | N   | N   | K+  | $\alpha$ 78       | 3         |
| L*            | I* | K+ | M*  | K+  | P   | $\alpha$ 206      | 1, 2      |
| N             | F* | K+ | S   | Q   | L*  | $\alpha$ 219      | 1         |
| E-            | W  | K+ | A*  | L*  | T   | $\alpha$ 230      | 1         |
| S             | Y  | K+ | M*  | A*  | D   | $\alpha$ 583      | 5         |
| T             | Q  | K+ | K+  | V*  | E-  | $\beta$ 53        | 3         |
| R+            | Q  | K+ | Q   | V*  | K+  | $\beta$ 130       | 2         |
| Q             | V* | K+ | D-  | N   | E-  | $\beta$ 133       | 4         |
| L*            | I* | K+ | A*  | I*  | Q   | $\gamma$ 62       | 4         |
| T             | L* | K+ | S   | R+  | K+  | $\gamma$ 85       | 2, 3      |
| S             | R+ | K+ | M*  | L*  | E-  | $\gamma$ 88       | 2         |

Ref. 1: Takagi T. and R.F. Doolittle, Biochemistry 14: 5149-5156, 1975; Ref. 2: Hantgan R.R., et al., Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition. Edited by R.W. Colman et al. J.B. Lippincott Company: Philadelphia, 1994; Ref. 3: Takagi T. and R.F. Doolittle, *supra*; Ref. 4: Nomura S. et al., Electrophoresis 14: 1318-1321 1993.; Ref. 5: Ständker L. et al., Biochemical and Biophysical Research Communications 215: 896-902 (1995).

\* Indicates a hydrophobic amino acid; +/- Indicates a charged side chain, either cationic (+) or anionic (-).

\*\* Single letter amino acid code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Given that plasmin is an important enzyme in cell migration and tissue/clot remodeling, these substrates or parts of these substrates represent useful sequences within the sites indicated above as XXXXX in P.

Likewise, collagenase is an important enzyme in cell migration and tissue remodeling. A study of the literature on collagenase indicates also a variety of substrate sites, which represent useful identities for XXXXX in P (Table 2; SEQ ID NOS: 25-31):



Table 2. Collagenase Substrate Sites found in Collagen

| P3 | P2 | P1 | P1' | P2' | P3' | Collagen type and site                                      | Ref. |
|----|----|----|-----|-----|-----|---|------|
| P  | Q  | G  | I*  | A*  | G   | calf & chick $\alpha 1(I)$ ; human cartilage $\alpha 1(II)$ | 6    |
| P  | Q  | G  | L*  | L*  | G   | calf $\alpha 2(I)$  | 6    |
| P  | Q  | G  | I*  | L*  | G   | chick $\alpha 2(I)$   | 6    |
| P  | Q  | G  | L*  | A*  | G   | chick $\alpha 2(I)$ ; human skin $\alpha 1(III)$            | 6    |
| P  | L* | G  | I*  | A*  | G   | human liver $\alpha 1(III)$                                 | 6    |
| P  | L* | G  | L*  | W   | A*  | human   | 7    |
| P  | L* | G  | L*  | A*  | G   | human   | 8    |

Ref. 6: Netzel-Arnett S. et al., The Journal of Biological Chemistry 266: 6747-6755, 1991; Ref. 7: Upadhye S. and V.S. Ananthanarayanan, Biochemical and Biophysical Research Communications 215: 474-482, 1995; Ref. 8: Liko Z., et al., Biochem Biophys Res Commun 227: 351-35, 1996.

The use of enzyme degradation sites within P, either in the nucleophile precursor component (most easy, since cysteine in the sequence may be used to provide a thiol as a nucleophile) or as the conjugated unsaturated precursor component, is that the rate of biomaterial resorption or remodeling may be linked to the rate and progress of healing, for example, as indicated by cell infiltration.

It is particularly powerful to note that the rate of biomaterial resorption may be modulated by adjustments to the oligopeptide sequence so as to alter the  $K_m$  and  $k_{cat}$  of the substrate site. For example, a study of the literature on the enzymology of collagenase substrate sites shows that it is possible to adjust the rate of degradation of substrates by the design of the sequence of the substrates (Table 3; SEQ ID NOS: 32-38):

Table 3. Design of Collagenase (Matrix metalloproteinase I)-Sensitive Peptide Sequences

| No. | Sequence | $k_{cat}/K_m$ relative to that of PQGIAG |
|-----|----------|--|
| 1   | GPQGIAGQ | 100% (normal)                            |
| 2   | GPVGIAGQ | 30% (slow)                               |
| 3   | GPQGVAGQ | 9% (slower)                              |
| 4   | GPQGRAGQ | <5% (very slow)                          |
| 5   | GPQGIASQ | 130% (fast)                              |
| 6   | GPQGIFGQ | >300% (faster)                           |
| 7   | GPQGIWGO | >700% (very fast)                        |

Netzel-Arnett S. et al., The Journal of Biological Chemistry 266: 6747-6755, 1991

### Accelerators

Poorly reacting nucleophiles are referred to as having a pseudo-first order half-life of more than approximately 15 minutes (with the conjugated unsaturated group present in excess; slower reactions might be useful in some medical circumstances), at a pH generally defined as pH more than 5 and less than 9, and at a temperature greater than 25°C and less than 40°C. Radical initiators are referred to as organic or water-soluble molecules undergoing spontaneous, thermally- or photochemically-initiated homolytic scission of carbon-heteroatom or heteroatom-heteroatom bonds, to produce carbon- or heteroatom-based radicals. The use of such radical initiators as accelerators, while not preferred, should be understood to be superior to polymerization, in that the concentration of free radicals employed can be much lower. The addition rate of poorly reacting nucleophiles to conjugated unsaturated groups can be enhanced by the presence of accelerating substances; these can be radical initiators, photosensitizers; (alone or in combination with radical initiators; Pathak, *supra*), low molecular weight Lewis acids (Pathak, *supra*), solid-state catalysts characterized by Lewis acidity or by the presence of quaternary ammonium ions, such as an Amberlyst resin (Pathak, *supra*) or a montmorillonite clay (Pathak, *supra*), or hydrogen bonding receptors, based on N,N-disubstituted urea or peptidic structures (Pathak, *supra*). In the last case, the acceleration mechanism is based on the stabilization by hydrogen bonding of the enolate-like

5 transition state, following the attack of the nucleophile on the conjugated olefin; tailor-made antibodies can be used on this purpose (Pathak, supra).

10 In a typical experiment a concentrated (typically greater than or equal to 10% w/w, but simply at a sufficiently high concentration to achieve the desired behavior) solution of  
5 a P derivative containing a number of conjugated unsaturated groups greater than one per P residue is quickly mixed with a concentrated (>10%, but simply at a sufficiently high  
15 concentration to achieve the desired behavior) solution of a thiol- or suitable amino-containing compound (especially thiols, in applications where the highest degree of self-selectivity may not be required), with a number of nucleophilic species greater than two.  
20 10 An accelerating species in catalytic quantities (<1-2% w/w) can be introduced during the mixing stage. Higher temperatures (up to 60°C) can be used for a short time after the mixing to activate the cross-linking reaction. For situations when the material is to be  
25 injected into the body and then allowed to react *in situ* to form the final biomaterial, injection temperatures up to approximately 50°C may be acceptable.

### 30 15 III. Polymer network formation Functionality

Utilizing terminology from polymer science, polymers can be made by reaction of  
35 monomers with a functionality of 2. Cross-linked networks of polymers can be made if some or all of the monomers have a functionality greater than 2. Molecules are described  
20 herein having a functionality greater than or equal to 2 (monomers or macromers), which can be reacted together to form a cross-linked network, where functionality is defined in  
40 terms of addition reactions. As used herein, polymerization refers to the reaction of monomers or macromers with functionality of 2, and cross-linking refers to the reaction  
of monomers or macromers some or all of which have a functionality greater than 2. The  
45 25 term monomers here is not limited to small molecules, but can also refer to polymers and

5 biopolymers.

10 The monomers described are of two classes, which when reacted together form a linear or cross-linked biomaterial. Both classes of monomers are required to be mixed together for cross-linking to occur (different approaches for mixing are described  
5 immediately below). One class of monomer contains 2 or more conjugated unsaturated groups (thus, a functionality of 2 or more), preferably conjugated. The other class of monomer contains 2 or more nucleophiles (thus, a functionality of 2 or more), preferably  
15 nucleophiles that are stronger nucleophiles than others present as other components of the system, for example, thiols when compared with amines that may be present as desirably non-reactive components of the system.

20 When water-soluble precursor monomers are mixed together (referred to as the final precursor solution), linear or cross-linked gels or networks are formed, and such reactions can proceed in water at physiologic or nearly physiologic salt concentrations  
25 and pH. It is not necessary that the monomers be entirely soluble in water, and indeed it is sometimes beneficial that they not be soluble in water. In such cases, gels may not be obtained as the final material, but rather more hydrophobic, less water-swelling materials. These can be particularly useful in the delivery of hydrophobic drugs and in the formation  
30 of materials with substantial structural strength. It is only necessary that the two components be either soluble in each other or at least finely dispersible in each other, perhaps in the presence of an emulsifying agent. In this manner, the two components can  
35 come close enough to each other to react to form the linear or cross-linked material.

40 It is also possible to work with solutions of monomers formed in a solution other than water. For example, the use of N-methyl pyrrolidone (NMP) as a solvent in injectable biomaterial systems is known, and as such it is possible, when one wishes to  
25 work with the precursor components in solution, but with precursor components that are not freely soluble in water, to employ certain organic solvents that are acceptable for use  
45

5 with the sensitive biological material under consideration.

When a drug is being incorporated in the laboratory or in a manufacturing line,  
then there is great flexibility in the selection of this organic solvent, since at least most of  
10 it will be removed before the implant is provided to the subject. When a material is being  
5 formed on the skin, then a great deal of flexibility also exists, due to the low skin toxicity  
of many organic solvents, including NMP, acetone, ethanol, isopropanol and ethyl  
15 acetate. When a material is being formed in the body, then the list of acceptable solvents  
is considerably smaller and is dominated by toxicity concerns. In such cases, NMP is a  
particularly favorable organic solvent. The toxicity of the solvent system can also be  
20 10 modulated by employing a mixed solvent system, comprising the organic solvent and  
water, to lower the overall concentration of organic solvent but to still provide good  
solubility or dispersability in the mixed solvent system.

25 Mixing to form the final precursor solution can occur by several means. Most  
straightforwardly, one solution contains the nucleophilic precursor component and one  
15 solution contains the conjugated unsaturated precursor component. These two  
components are formulated in solvent and buffer systems such that the pH and  
30 concentrations obtained after mixing are appropriate for the chemical reaction to proceed.  
Such mixing could occur in a static mixer at the function of two syringes, for example.

35 Other mixing approaches can be imagined. For example, mixing can occur  
20 between fine particles of each of the two precursor solutions in an air spray. One solution  
could be prepared from both precursor components, but at a pH, for example, such that  
the reaction did not proceed or proceeded only slowly. After placement of the pre-mixed  
40 precursor solution, pH could be adjusted (e.g., by change of temperature, or mixing with  
acid or base, or by a chemical reaction to create an acid or base, or diffusion of an acid or  
25 base), to result in a final condition in the final precursor solution that was appropriate for  
45 the chemical reaction to proceed. Another approach can be to prepare the final precursor

5 solution at a temperature such that the reaction did not proceed or proceeded only very  
slowly, either related to the activation energy of the reaction or to a buffer with  
10 temperature-sensitive characteristics or both. Upon warming or cooling (most usefully  
warming) to the final application temperature (e.g., to body temperature after injection),  
5 the conditions in the final precursor solution would be appropriate for the chemical  
reaction to proceed.

### 15 Medical applications

Since the biomaterials are useful as medical implants or devices, or for drug  
20 delivery in humans, the system of molecules used in the precursor solution must meet  
certain criteria. These include:

1. The rate of the Michael-type reaction must occur over a clinically relevant  
25 period of time at a clinically relevant temperature and pH. Generally, gelation over a  
period of less than approximately 15 minutes, at a pH generally more than 7 and less than  
9 and at a temperature greater than 25 and less than 40°C is desirable.

15 2. The reaction must be sufficiently self-selective, with self-selectivity  
30 considerations including the following. For the formation of gels in the presence of drugs  
containing amines or where reaction with cell and tissue components is undesirable, the  
conjugated unsaturation must react very slowly with amines at the pH of application of  
35 the final precursor solution. Preferably, a ratio of reactivity of the conjugated  
20 unsaturation for the nucleophile of intentional reactivity to the amine, in this case the  
nucleophile of unintentional or undesirable reactivity, in excess of ten and more  
40 preferably even higher is desired. Typically, the approach of Michael-type addition  
between conjugated unsaturations and thiols will not be useful for drugs that contain  
themselves conjugate unsaturations or thiols. Exceptions include cases when the  
45 25 reactivity of the group on the drug is considerably less than the reactivity on the

5 corresponding group in the biomaterial precursor and cases when such reactions are not detrimental, for example, when grafting to the biomaterial network are not detrimental.

10 3. The reactants must be stable in water, when the precursor solutions are prepared in water. Stable is defined as reacting slowly, with slowly defined as sufficiently slow to allow the reaction between the two components to proceed and still result in the formation of the desired biomaterial.

15 4. The addition reaction in the final precursor solution must not be exothermic to the point of causing tissue damage, drug breakdown or other detrimental results to the biological material under consideration. The temperature of the gelling solution generally should not be raised above 60°C during gelation, and preferably even cooler maximum reaction temperatures are desirable.

20 10 5. The components of the precursor solution must not be toxic at concentrations which diffuse out of the final precursor solution as it is applied, with the word toxic being defined as inducing a medically unacceptable tissue reaction in a medically relevant context.

25 15 The criteria defined above in this section limit the identity of the molecules which may be useful in the precursor solution, by limiting the identity of the chemical group used for the cross-linking.

#### 35 Additional Biofunctionality

20 One strong benefit of the use of the addition reactions described herein is that other bioactive biofunctional groups can be incorporated into the biomaterial, for example, to provide sites for binding of adhesion-promoting receptors on the cell surface or sites for growth factor binding.

*Adhesion peptides*

A variety of adhesion-promoting peptides have been identified as being the active domains of adhesion-promoting proteins such as fibronectin, vitronectin, laminin, collagen, von Willebrand factor, osteonectin, and so forth. These peptides can be readily incorporated into the biomaterial, when they are designed with a strong nucleophile in the peptide chain, such as a cysteine. Such an example is demonstrated in the examples. A partial list of the peptides that would be of interest follows (Table 4; SEQ ID NOS: 39-49):

**Table 4. Cell Binding Domain Sequences of Extracellular Matrix Proteins**

| Protein        | Sequence  | Role   |
|----------------|-----------|--|
| Fibronectin    | RGDS      | Adhesion of most cells, via $\alpha_5\beta_1$      |
|                | LDV       | Adhesion   |
|                | REDV      | Adhesion   |
| Vitronectin    | RGDV      | Adhesion of most cells, via $\alpha_v\beta_3$      |
| Laminin A      | LRGDN     | Adhesion   |
|                | IKVAV     | Neurite extension                                  |
| Laminin B1     | YIGSR     | Adhesion of many cells, via 67 kD laminin receptor |
|                | PDSGR     | Adhesion   |
| Laminin B2     | RNIAEIKDA | Neurite extension                                  |
| Collagen I     | RGDT      | Adhesion of most cells                             |
|                | DGEA      | Adhesion of platelets, other cells                 |
| Thrombospondin | RGD       | Adhesion of most cells                             |
|                | VTXG      | Adhesion of platelets                              |

After Yamada, Y., and Kleinman, H.K., *Curr. Opin. Cell Biol.* 4:319, 1992.

These peptides are potentially useful in controlling a variety of cellular reactions, such as cell attachment, migration and overgrowth upon a material surface (especially when the material is not degradable or is slowly degradable), cell migration through a material (especially when the material is more readily degradable by the incorporation of protease substrates within one of the two precursor components), and the induction of



particular cellular phenotypes (e.g., stimulating a macrophage to release beneficial growth factors but not to form foreign body giant cells). The peptides shown in Table 5 (SEQ ID NOS: 50-57) bind to cell surface receptors that are glycoproteins. There are other such peptide sequences that bind to cell-surface heparan-sulfate and chondroitin-sulfate containing proteoglycans, called as a family heparin-binding peptides. These can also be incorporated to confer cell adhesion via binding to such cell-surface components.

Table 5. Proteoglycan Binding Domain Sequences of Extracellular Matrix Proteins

| Protein                      | Sequence                      |
|------------------------------|-------------------------------|
| $\chi$ BB $\chi$ B $\chi$ *  | Consensus sequence            |
| PRRARV                       | fibronectin                   |
| YEKPGSPPREVVPRPRPGV          | fibronectin                   |
| RPSLAKKQRFRRHNRKGYRSQBGHSRGR | vitronectin                   |
| RIQNLLKITNLRKFVK             | laminin                       |
| K( $\beta$ )FAKLAARLYRKA     | antithrombin III              |
| KHKGRDVILKKDVR               | neural cell adhesion molecule |
| YKKIKKL                      | platelet factor 4             |

References for first five entries given in Massia, S.P., and Hubbell, J.A. J. Biol. Chem. 267:10133-10141, 1992; Antithrombin III sequence from Tyler-Cross, R., et al., Protein Sci. 3: 620-627, 1994; Neural cell adhesion molecule sequence from Kallapur, S.G., and Akeson, R.A., J. Neurosci. Res. 33: 538-548, 1992; Platelet factor 4 sequence from Zucker, M.B., and Katz, I.R., Proc. Soc. Exp. Biol. Med. 198, 693-702, 1991.  
 \* $\chi$  indicates a hydrophobic amino acid. Basic amino acids are shown underlined.

It should be noted that the practical method for incorporation of the adhesion peptide by the method of the present invention is much easier than the state of the art (Pathak, *supra*). By such method as used in the prior art (taking the example of the formation of a Peptide-PEG-Acrylate), a heterobifunctional PEG must be synthesized, with an activated ester on one end and an acrylate on the other end. This must be grafted to the peptide, and purified. This agent is then useful for either incorporation by the method of this invention or by polymerization of the acrylate end groups, for example in a PEG diacrylate as taught by Hubbell et al. By contrast, the present method of peptide

incorporation is much easier. The nucleophile (e.g., cysteine with a free thiol) containing peptide is simply mixed with the PEG diacrylate (or the multifunctional PEG conjugate unsaturated structure), is allowed to react for a short period of time, and then either the remainder of a different multinucleophile is added or the system is photopolymerized. There is no synthesis of a heterobifunctional agent, and there is no purification after coupling. This is possible due to the self-selectivity of the system.

#### *Growth factor binding peptides*

A second sort of biofunctionality that is useful in biomaterials are structures that bind growth factors. These can be used in the controlled delivery and release of growth factors. An excellent example can be found for heparin-binding growth factors, which include aFGF, bFGF, VEGF, TGF $\beta$ , and BMP. It is straightforward to incorporate peptides that bind heparin (as described above, and further below). Heparin can be added to this mixture, along with the growth factor. Because of the self-selectivity of the system, chemical reaction with the heparin and the growth factor would not be expected to occur. Thus, if a heparin-binding peptide containing a single free thiol at a cysteine residue were mixed with heparin and a heparin-binding growth factor, and if these components were mixed with, for example, a PEG-triacrylate, and if this were mixed with a protease substrate peptide with two thiols by the incorporation of two cysteine residues (one each on both sides of the substrate domain), the following biomimetic biomaterial would result: the biomaterial would be degradable by cell-associated proteases, and the growth factor would be bound into the biomaterial by non-covalent binding to heparin, which is in turn non-covalently bound to the heparin-binding peptide, which is, in turn, covalently bound to the hydrogel biomaterial. Alternatively, one could functionalize heparin directly so that it contains a single strong nucleophile and is directly chemically bound into the polymer network. Another related way to sequester heparin-binding

5 growth factors would be more directly through the use of covalently incorporated heparin mimics (c.g., peptides with negatively charged side chains) that directly bind growth factors.

10 There now follow particular examples that describe the preparation of compositions of the invention, and the methods of the invention. These examples are provided for the purpose of illustrating the invention, and should not be construed as limiting.

### Example 1: Preparation of Basic Reagents

#### Acrylation of poly(ethylene glycol) diol

10 Polyethylene glycol, mol. wt. 8000 (50 g, 6.25 mmol, 12.5 mmol hydroxyl groups, Aldrich, Milwaukee, WI, USA) was added to toluene (500 ml) and dried by azeotropic distillation. The mixture was cooled to 0°C and 100 ml of anhydrous dichloromethane 25 (Aldrich) was added. Triethylamine (2.61 ml, 18.75 mmol, 1.5 eq. based on hydroxy groups, Aldrich) was added, followed by dropwise addition of acryloyl chloride (2.03 ml, 18.75 mmol, 1.5 eq., Aldrich). The reaction was kept under Ar overnight in the dark. 30 15 The product was filtered and then recovered by precipitation in hexane with stirring. The product was redissolved in 75 ml of dichloromethane and precipitated again in hexane with stirring. The product was dried overnight under vacuum. The product was dissolved 35 in 500 ml of water with 25 g NaCl, and the pH was adjusted to pH 6. The solution was extracted with dichloromethane (Aldrich) 3 times (the first extraction with 20 dichloromethane should not be shaken vigorously to avoid the formation of an emulsion). The dichloromethane fractions are combined and added to stirring hexane. The product is recovered by filtration and dried under vacuum overnight. By <sup>1</sup>H-NMR, 80% of alcohols 40 on the polyethylene glycol are acrylated (product is referred to as polyethylene glycol diacrylate) 45 25

**Acrylation of poly(ethylene glycol) triol**

PEG-triacrylate (PEG-3A) is a three-armed PEG with glycerol core. Molecular weight notations (PEG-2500-3A, PEG-3500-3A) refer to total average molecular weight and not to the molecular weight of a single arm. The acrylation was carried out using exactly the same molar ratios of reactants as described for the PEG diol.

**Crotonylation and dimethylacrylation of poly(ethylene glycol) diol**

Crotonoyl PEG-8000 (PEG-8000-2C) (crotonyl,  $-\text{OOC}-\text{CH}=\text{CHCH}_3$ ) and dimethacryloyl PEG-8000 (PEG-8000-2DMA) (dimethylacryloyl,  $-\text{OOC}-\text{CH}=\text{C}(\text{CH}_3)_2$ ) were synthesized simultaneously in side by side reactions. 27 g PEG-8000 (3.375 mmol, 6.75 mmol hydroxyl groups; Aldrich) was dissolved in benzene and azeotropically distilled until the distillate appeared clear. The PEG-benzene solution was allowed to cool to room temperature. Then 100 ml of the solution was anhydrously transferred to a separate round bottom flask. Triethylamine (1.1 ml to the 100 ml sample and 1.7 ml to the larger (150 ml) sample, 3 equivalents based on hydroxyl groups; Aldrich) was added to each flask. Crotonoyl-Cl (1.2 ml, 3 equivalents based on hydroxyl groups; Fluka) was added dropwise to the 150 ml sample. Dimethacryloyl-Cl (0.9 ml, 3 equivalents based on hydroxyl groups; Fluka) was added dropwise to the 100 ml sample. The reactions were run 20 hours in the dark. The solutions were filtered through paper and precipitated in hexane. Both precipitates were dried in vacuo. The degrees of modification were determined by  $^1\text{H}$  NMR to be 85% for the PEG-8000-2C and 89% for the PEG-8000-2DMA (by degree of esterification).

**Preparation of bis(benzoquinones) PEG*****STEP A) Preparation of bis-carboxyl PEG***

17 g (5 mmol) of 3400 PEG diol are dissolved in 500 ml of toluene and dried by

azeotropic distillation: 15 ml of 1M THF solution of potassium *tert*-butoxide (15 mmol) are added and the reaction mixture is refluxed for 10 minutes, then cooled to room temperature. 5.4 ml (50 mmol) of ethyl 2-bromoacetate are then added: the solution is stirred for 24 hours at 40°C, then filtered to remove KBr, concentrated at the rotary evaporator and precipitated in cold diethyl ether. The solid is then dissolved in 250 ml of 0.2 N NaOH (the pH is kept at 12 by dropwise addition of 4 N NaOH); the solution is stirred for 12 hours, and after the pH drops to 4 by dropwise addition of concentrated HCl, is extracted with dichloromethane; the organic phase is dried on sodium sulfate and precipitated in cold diethyl ether. The degree of modification is determined by <sup>1</sup>H-NMR.

10 *STEP B) Preparation of bis(carboxyl 2,5-dimethoxyanilide) PEG*

10 g (2.8 mmol, 5.7 meq) of 3400 bis-carboxyl PEG are dissolved in 200 ml of THF together with 2.0 g (6 mmol) of 2,5-dimethoxyaniline (recrystallized three times from hexane); 0.73 g (5.8 mmol) of diisopropyl carbodiimide are then added and the solution is stirred for 24 hours at room temperature. The precipitated diisopropyl urea is filtered off, the THF evaporated at the rotary evaporator; the polymer is then redissolved in toluene, the solution is filtered and then precipitated in cold diethyl ether. The procedure is repeated twice. The degree of modification is determined by <sup>1</sup>H-NMR.

35 *STEP C) Preparation of bis(carboxyl 2,5-hydroxyanilide) PEG*

5 g (1.4 mmol, 5.2 meq) of 3400 bis (carboxyl 2,5-dimethoxyanilide) PEG are dissolved in 50 ml of dry dichloromethane in dry nitrogen atmosphere; 1.2 g (6 mmol, 0.82 ml) of iodotrimethylsilane are then added and the solution is stirred for 24 hours at room temperature. The dichloromethane solution is then washed with water till neutrality, dried over sodium sulfate, concentrated to small volume and precipitated in hexane. The reaction yield is determined by <sup>1</sup>H-NMR.

**STEP D) Preparation of bis(carboxamide 2,5-benzoquinones) PEG**

5 g (1.4 mmol, 5.6 meq) of 3400 bis(carbonyl 2,5-hydroxyanilide) PEG are dissolved in 50 ml of ethanol and 1.2 g (7.4 mmol) of iron (III) chloride. The solution is stirred for 24 hours at room temperature, then 150 ml of dichloromethane and 150 ml of water are added and two phases separate; the dichloromethane phase is washed three times with water, then concentrated and precipitated in cold diethyl ether. The reaction yield is determined by <sup>1</sup>H-NMR.

**Preparation of  $\alpha,\omega$ -bis(benzoquinones) poly(lactic acid)-PEG-poly(lactic acid) block copolymer (example with 2.5 monomeric units of lactic acid per PEG end)****STEP A) Preparation of poly(lactic acid)-PEG-poly(lactic acid) block copolymer**

17 g (5 mmol) of dry 3400 PEG diol, 3.60 g (0.025 mol) of dl lactide and 15 ml of stannous octanoate are mixed together under dry nitrogen atmosphere. The reaction mixture is stirred at 200°C for 2 hours and at 160°C for 2 hours and subsequently cooled to room temperature. The resulting solid is dissolved in dichloromethane and precipitated in cold diethyl ether.

**STEPS B to E**

Steps B to E are analogous to the steps A to D in the preparation of bis(benzoquinones) PEG.

**Preparation of poly(ethylene-co-vinyl alcohol-co-2-oxyvinyl-(2',5'-benzoquinones)acetamide)****STEP A to D) Preparations of poly(ethylene-co-vinyl alcohol-co-2-oxyvinyl-acetic acid)**

These preparations are analogous to the STEPS A to D in the preparation of bis(benzoquinones) PEG.

**Preparation of bis(4-vinylpyridyl) PEG**

10 g (2.7 mmol) of freshly prepared 3400 PEG triflate were reacted for 24 hours at 0°C with 0.75 g (8 mmol) of 4-vinyl pyridine in 30 ml of dry NMP. The solution was precipitated in cold diethyl ether, the solid redissolved in dichloromethane and precipitated again in cold diethyl ether.

**Peptide synthesis**

Peptides were synthesized on a Perseptive Biosystems (Framingham, MA, USA) Pioneer peptide synthesizer using the standard Fmoc protection scheme. Peptides were cleaved from the resin using 8.8 ml trifluoroacetic acid (Perseptive Biosystems). 0.5 ml of water, 0.5 ml phenol (Aldrich), and 0.2 ml triisopropylsilane (Aldrich) per gram of resin, for 2 hr at room temperature. The solution was precipitated in ether, and product recovered by filtration, and dried under vacuum. Peptides were purified by C18 chromatography, and fractions containing product were identified by MALDI-TOF mass spectrometry. Peptides were stored under Ar at -20°C.

Prior to application, cysteine-containing peptides were handled wet in acidic solutions and/or degassed solutions, or dry under vacuum or under argon as much as possible to prevent oxidation.

**Example 2: Gel formation by conjugate addition reactions**

**Gels formed by conjugate addition with a low molecular weight tri-thiol and a PEG-linked unsaturation: trimethylolpropane tris(3-mercaptopropionate) and PEG diacrylate**

50 mg PEG-8000-2A was dissolved at 0.1 g/ml in 500 microliters of 4:1 50 mM bicarbonate buffer (pH 8.4): acetonitrile. 1.1 microliters of trimethylolpropane tris(3-mercaptopropionate) (1.25 equivalents based on acrylates) were added and the solution

5 mixed by vortexing. The trimethylolpropane tris(3-mercaptopropionate) was not  
perfectly miscible in the solution but formed a suspension of small droplets in the  
aqueous phase. The material did not gel in two hours but was let to sit overnight. At  
10 approximately 12 hours after addition of the trimethylolpropane tris(3-  
5 mercaptopropionate), a solid cross-linked material had formed. Water was added to the  
material, which swelled with the water but did not dissolve (time scale: weeks before gel  
15 finally discarded due to contamination).

Likewise, a gel of higher concentration of PEG and with stronger mechanical  
properties was formed by first dissolving 0.2 g PEG-8000-2A in 750 microliters of  
20 10 unbuffered water and 250 microliters acetone. 4.4 microliters of trimethylolpropane  
tris(3-mercaptopropionate) (1.25 equivalents based on acrylate groups) were added.  
While trimethylolpropane tris(3-mercaptopropionate) is soluble in acetone at these  
25 concentrations (4.4 microliters trimethylolpropane tris(3-mercaptopropionate)/250  
microliters acetone), it still formed an visibly insoluble suspension with the PEG solution  
15 upon vortexing. After 2- 4 hours, a highly cross-linked water insoluble material had  
formed.

**Gels formed by conjugate addition with a peptide-linked nucleophile and a PEG-  
linked conjugated unsaturation**

35 The peptide GCYKNRDCG (SEQ ID NO: 58) was designed to be sensitive to  
20 hydrolysis by the enzyme plasmin, to contain more than one thiol (cysteine) for addition  
reaction with conjugated unsaturated groups, and to be very water soluble. The peptide  
40 was synthesized according to the methods described above. The peptide was extremely  
water soluble, up to at least 120 mg/ml.

Gels were formed from PEG-2500-3A and GCYKNRDCG as well as from PEG-  
45 25 3500-3A and GCYKNRDCG. Gels have been formed at three ratios of acrylates to



5           sulfhydryls (1: 1, 1.1: 1, and 1.25: 1). Gels were formed in 10 mM phosphate buffered  
saline with triethanolamine to adjust the pH to 8.0-9.0 as tested by paper pH strips (gel  
10           formation reactions were performed at 50 microliter and smaller scales). Gels have been  
made by: predissolving the peptide and then adding peptide solution to PEG-3A; by  
15           predissolving the PEG-3A and adding its solution to the peptide; and by predissolving  
both solutions and then mixing them in appropriate ratios.

15           The following protocol has been used for gel formation at the 40 microliter scale.  
The amount of PEG-2500-3A weighed into an Eppendorf varies due to the sticky  
qualities of the material that make is somewhat difficult to maneuver. However, the  
20           buffer volume added to the PEG-2500-3A is always adjusted to give the same final  
concentration on a mass/volume basis.

25           2.5 mg of GCYKNRDCG were weighed into an Eppendorf tube. 7.0 mg of PEG-  
2500-3A were weighed into a separate Eppendorf tube. 62 microliters of phosphate  
buffered saline (PBS)•TEA (10 mM PBS with 13 microliters of triethanolamine/ml)  
15           were added to the PEG-2500-3A to give a solution of 4.5 mg/40 microliters. The PEG  
solution was allowed to sit until the PEG-3A had dissolved (less than five minutes). 40  
30           microliters of the PEG-3A solution were added to the peptide, which dissolved extremely  
rapidly. The pipet tip used for the transfer was used to stir the mixture for approximately  
3 seconds. A 1 microliter sample was withdrawn to test the pH by a paper strip (pH range  
35           1-11). The pH was approximately 8.0. After 20-30 minutes, a gel had formed.

**Controlling the rate of gelation by modulating charge near a nucleophile (e.g., thiol)**

40           Two collagenase (MMP-1) sensitive peptides were synthesized:  
GCDDGPQGIWGQDDCG (SEQ ID NO: 59) and GCRDGPQGIWGQDRCG (SEQ ID  
NO: 60) using standard Fmoc techniques described in Example 1. In one peptide, the  
45           thiol (in cysteine, C) was close to a residue bearing negative charge (aspartic acid, D)

when near neutral pH, the pH of interest. In the other peptide, the thiol was near a residue bearing positive charge (arginine, R) when close to neutral pH. Each peptide was reacted separately with acrylate containing polymers of PEG at pH 8. The rate of conjugate addition was followed by the consumption of thiols by using DTNB, Ellman's reagent. Results are shown in Figure 1. The exchange of D -> R (negative charge -> positive charge) next to the thiol increased the rate of reaction such that the half life of thiol consumption during gel formation was decreased almost 3 fold. This was accomplished by design in order to increase the likelihood that the thiol exists in the S- form which participates in the conjugate addition and thus to increase the rate of reaction and gelation.

#### Swelling (water content) of gels made by conjugate addition

Gels were made with 0.1 g/ml, 0.15 g/ml, and 0.2 g/ml PEG-2500-3A at a 20 microliter scale. The gels contained 1.1 acrylates per sulfhydryl in the peptide (nucleophile) component, GCYKNRDCG. For gel formation, PBS buffers were adjusted to account for added acidity of additional peptide in higher concentration gels and to give reactions at pH 8.0-8.5. Gels were made in quadruplicate.

Table 6. Conjugate addition gels for swelling studies

|     | PEG-2500-3A<br>(mg) | GCYKNRDCG<br>(mg) | Triethanolamine<br>( $\mu$ l/ml) | % water in swollen<br>gel |
|-----|---------------------|-------------------|----------------------------------|---------------------------|
| 10% | 2.0                 | 1.1               | 13.0                             | 96.5%                     |
| 15% | 3.0                 | 1.7               | 20.1                             | 95.8%                     |
| 20% | 4.0                 | 2.2               | 26.0                             | 94.8%                     |

Gels were swollen in 10 mM PBS, pH 7.3 for 48 hours before the first wet weight measurements were made. Gels were weighed wet four times over three consecutive days with no significant increase in wet masses over this time. Then the gels were soaked in

5 deionized water, with exchanges of the solution phase, for four days after which time the  
gels were lyophilized in order to obtain dry masses. Water contents based on the  
maximum possible dry masses (due to variability in actual dry masses) are all  
10 approximately 95% by mass of the swollen gels.

5 **Gels formed by mixing two powder components**

15 Material precursors may also be delivered to tissues in the dry state. For example,  
PEG dithiol can be formulated as a powder, PEG tetraacrylate can be formulated as a  
powder, one or the other but preferably both components containing buffer components  
20 such that when the two powder components are mixed and dissolved in water, saline or  
physiological fluids a solution forms at a pH such that reaction of the two precursor  
10 components occurs, e.g. pH 8. These powder components may be sprayed upon a tissue  
surface, either along with an aqueous stream or without one. In the case that the powders  
25 are sprayed with an aqueous stream or without one. In the case that the powders are  
sprayed with an aqueous stream, the polymer components dissolve in the aqueous stream,  
15 along with the layer of biological fluids on the tissue surface, and then react to form the  
final biomaterial implant. In the case of application of the powder components to the  
30 tissue surface, the polymeric precursors and the buffer components dissolve in the  
biological fluids and form a precursor solution, capable of reaction to form the final  
35 biomaterial implant. In the case where the biological fluids provide the moisture for  
dissolution of the polymeric precursor components. the concentration of the polymeric  
20 precursor components may be high, resulting in a strong biomaterial implant and good  
adhesion to the tissue. In the application of the powder streams, the powders may be  
40 mixed together and then applied as a single powder mixture to the moist tissue surface, or  
they may be mixed in a spray from two components. The powder components may be  
45 25 formed by methods know to those skilled in the art of powder technology, such as

precipitation, grinding, milling, lyophilization, spray drying, and so forth. Small particles will lead to more effective and rapid dissolution, either in an aqueous stream or in moisture at the tissue surface. The two polymeric precursor components should be mixed together at a ratio such that the thiol components and the acrylate components are approximately equi-equivalent on a mole of thiol per mole of acrylate basis. Furthermore, the nature of the biomaterial implant may be controlled by adding other agents to the precursor powders, such as particles that are slow to dissolve in aqueous solution or gas formers, both of which will lead to the formation of pores within the material implant after curing.

**Example 3: General protocols for immobilizing peptides and testing activity with cells in culture**

**Peptide immobilization in gels in which the peptide is immobilized by conjugate addition and the gel is formed by conjugate addition**

13.9 mg PEG-2500-3A was dissolved in 69.5 microliters (5.0 mg/25 microliters) of PBS•TEA (10 mM PBS containing 13 microliters of triethanolamine/ml) containing GCGYGRGDSPG (SEQ ID NO: 61) at a concentration of 3.2 mg GCGYGRGDSPG/ml. 7.0 mg GCYKNRDCG was dissolved in 65 microliters of PBS•TEA (2.7 mg/25 microliter). The GCYKNRDCG was filtered through a 0.22 micron filter. After 9 minutes of reaction time, the PEG-2500-3A/GCGYGRGDSPG solution was separately filtered through a 0.22 micron filter. As soon as the filtrations were complete, equivolumes (25 microliters) of the two solutions were added to wells of a Corning flat-bottomed tissue culture treated polystyrene 96 well plates. As the second of the two precursor solutions was added, the pipet tip was used to stir the mixture for 2-3 seconds. Then the gels were allowed to set at 37°C.

**Cell-resistance of gels made by conjugate addition lacking incorporated adhesion peptides**

Conjugate addition gels were made with 0.1 g/ml PEG-2500-3A and 1.1 acrylates per sulfhydryl in GCYKNRDCG. The gels were swollen for 24 hours in Dulbecco's modified Eagle's medium (some in serum-free conditions and some in 10% fetal bovine serum) containing 1% antibiotic and antimycotic agents. Human foreskin fibroblasts (passage 7; passaged with trypsin/EDTA) were seeded onto the gels. From time points two hours to 48 hours, the cells remained round and did not spread. The cells became increasingly clumped together. The cellular behavior was independent of serum in the medium. Control cells seeded on tissue culture treated polystyrene spread normally.

**Cell interaction with gels made by conjugate addition containing incorporated adhesion peptides**

Conjugate addition gels were made with PEG-2500-3A, GCYKNRDCG, and an RGD-containing peptide (GCGYGRGDSPG) incorporated in a pendant fashion. The gels were made with 0.1 g PEG-2500-3A/ml and 1.1 acrylates per sulfhydryl in GCYKNRDCG. The gels were swollen for more than 36 hours in Dulbecco's modified Eagle's medium (some in serum-free conditions and some in 10% fetal bovine serum) containing 1% antibiotic and antimycotic agents. When the RGD peptide was incorporated on one of every 12 acrylates of the PEG-2500-3A, human foreskin fibroblasts (passage 8; passaged by trypsin/EDTA) adhered to the gels (both those swollen in serum-free conditions and those in serum-containing medium). At 6 hours post-seeding, the cells were uniformly distributed over the gel surface, and approximately 50% of the seeded cells were spread (in both medium conditions).

**Cell interactions with the polyethylene glycol networks**

Cell interactions with the polyethylene glycol networks were tested by seeding human cells onto the gels using standard tissue culture methods. Human foreskin fibroblasts or human umbilical vein endothelial cells were purchased from Clonetics (San Diego, CA, USA). Fibroblasts were cultured in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum and 1% antibiotics (all from GIBCO BRL, Grand Island, NY, USA) at 37°C and 5% CO<sub>2</sub>. Endothelial cells were cultured in M199 media with 10% fetal bovine serum and 1% antibiotics (all from GIBCO BRL). Per 50 ml of media were added 100 µg/ml heparin (Sigma, St. Louis, MO, USA) and 3 mg of Endothelial cell growth supplement (Becton Dickinson Labware, Bedford, MA, USA). Cells were removed from culture substrates using Trypsin/EDTA (GIBCO BRL), centrifuged (500 g for 5 min for fibroblasts, 250 g for 5 min for endothelial cells), and resuspended in the normal cell culture media before seeding onto polyethylene glycol gels.

**Example 4: Chemical analysis of reaction products****Reaction kinetics measured with Ellman's reagent**

Ellman's reagent was used to measure the concentration of thiols in a solution. The assay utilized a solution of 40 mg Dinitrodisthiol nitrobenzene (Sigma) in 10 ml of 0.1 M phosphate buffer pH 8 (Ellman's reagent). A solution was tested for the presence of thiols by the addition of the solution to 3 ml of the phosphate buffer. Ellman's reagent (100 µl) was added and mixed. After 15 min, the absorbance of the solution was measured 412 nm. A molar absorption coefficient of 14150 was assumed.

Using the amino acid cysteine, Ellman's reagent revealed no detectable disulfide bond formation at pH 10 within 30 min at room temperature. If cysteine were added to an excess of PEG diacrylate, mol. wt. 8000, at the same conditions, the concentration of

thiols dropped to 0.2% of the original value within seconds, and did not decrease further out to 30 min. demonstrating the rapid disappearance of thiols in the presence of PEG diacrylate. The conjugate addition reaction between PEG diacrylate and the amino acid cysteine is shown in Figure 2.

The peptide with the amino acid sequence Ac-GCGYGRGDSP-NH<sub>2</sub> (SEQ ID NO: 62) was tested similarly. PEG diacrylate was dissolved in the phosphate buffer at pH 8 at a concentration of 25  $\mu$ mol in 1 ml. The peptide (1  $\mu$ mol) was added to the PEG diacrylate solution, and the disappearance of thiols was monitored using Ellman's reagent (see Figure 3). The reaction was performed at different pH, and additionally, the formation of disulfide bonds was assessed by dissolving the peptide at the same concentrations but in the absence of PEG diacrylate. The half life for the reaction was about 3 min at pH 7.4, and only a few seconds at pH 8, at room temperature.

Another method to on-line follow the reaction between thiols and PEG diacrylate is monitoring the absorbance of the reaction mixture at 233 nm. At this wavelength, the absorbance is in principle due to four substances: the thiol components, the disulfide impurities in the thiol component, the acrylate and the product (the  $\beta$ -alkylthio propionic ester). The experiments were conducted in 10<sup>-3</sup> M PBS buffer at various temperatures between 20°C and 37°C.

Performing experiments at several reactants ratios, but keeping constant the overall molar concentration of reactive groups (Figure 4), the extinction coefficients of all the species can be calculated fitting the absorption values before and after the reaction. This procedure allowed to follow independently the time evolution of the concentration of reactants and products: PEGDA and cysteine showed a single exponential behavior, with same kinetic constants and this proofed the reaction to be first order for every reactant. Half-life times between 2 and 10 minutes, depending on temperature and reactants concentration have been recorded. In Table 7. the kinetic constants are listed.

Table 7: First-order kinetic constants for PEGDA-cysteine reaction at different PEGDA content, with a  $2.5 \times 10^{-3}$  M overall cumulative concentration

| PEGDA equivalent fraction | k (min <sup>-1</sup> ) |
|---------------------------|------------------------|
| 0.18                      | 0.14                   |
| 0.33                      | 0.12                   |
| 0.46                      | 0.06                   |
| 0.57                      | 0.10                   |
| 0.75                      | 0.28                   |
| 0.82                      | 0.32                   |
| 0.88                      | 0.42                   |
| 0.95                      | 0.59                   |

Reaction of the PEG diacrylate with primary amines was also assessed. PEG diacrylate was mixed with a peptide with the amino acid sequence GDGSGYGRGDSPG (SEQ ID NO: 63), which contains only one primary amine at the amine terminus of the peptide. The presence of amines was measured using the fluorescamine assay. Fluorescamine (Sigma) was dissolved in dry acetone at 1.5 mg/ml. The peptide (1 mg) was added to 100  $\mu$ l of 0.1 M phosphate buffer at pH 8. PEG diacrylate, mol. wt. 8000 (100 mg), was dissolved to 900  $\mu$ l in 0.1 M phosphate buffer, pH 8 and mixed with the peptide solution. Samples were taken from the reaction and added to 100  $\mu$ l of 1.5 mg fluorescamine in dry acetone, and raised to 1 ml with 50 mM borate buffer at pH 9.

The fluorescence intensity was measured using a spectrofluorimeter, and concentrations calculated by comparison with a standard curve produced using the amino acid glycine. The half life for the reaction of the amine with an acrylate was about 90 min at pH 8 and 37°C.



**Production of PEG-peptide adducts assessed using size exclusion chromatography**

Aqueous size exclusion chromatography was performed using a Shodex OHpak SB-803 column (Showa Denko, Tokyo, Japan), using UV detection, measuring absorbance from 200-400 nm. The eluent was phosphate buffered saline (10 mM sodium phosphate, 140 mM NaCl, pH 7.4). PEG diacrylate has maximum absorbance at 205 nm, whereas the peptide used, GCGYGRGDS (SEQ ID NO: 64) has absorbance maxima at 220 and 270 nm, due to the presence of amide bonds and a tyrosine. PEG diacrylate was dissolved in 0.1 M phosphate buffer at pH 8 at a concentration of 25  $\mu$ mol in 1 ml. A sample of the solution was separated using size exclusion chromatography, and the polyethylene glycol eluted as a single peak with an absorbance maximum at 205 nm, and no absorbance at 220 or 270 nm. Next, the peptide (12.5  $\mu$ mol) was added to the PEG diacrylate solution, and reacted at room temperature for 5 min. A sample was then separated using size exclusion chromatography, and a single peak was detected, with absorbance maxima at 205, 220, and 270 nm, with the same retention time as PEG diacrylate. This indicated that the peptide reacted with the PEG diacrylate. Similar studies were performed using C18 chromatography, using a gradient from 95% water with 0.1% trifluoroacetic acid, 5% acetonitrile to 40% water with 0.1% trifluoroacetic acid, 60% acetonitrile. The peptide Ac-GCGYGRGDSP-NH<sub>2</sub>, eluted at about 20% acetonitrile, whereas PEG or PEG-3400 diacrylate eluted at about 40% acetonitrile. Incubation of 1 mol of the peptide per 2 mol of PEG-3400 diacrylate in buffered water at pH 8 led to the disappearance of the peptide-related peak that elutes at 20% acetonitrile, with the emergence of absorbance bands at 220 and 270 nm that coeluted with the PEG peak at 40% acetonitrile. Collecting the peaks and analyzing by MALDI-TOF mass spectrometry indicated that the PEG-associated peak contained a mixture of unmodified PEG-3400 diacrylate, and a new species with molecular weight that was the sum of the PEG-3400 diacrylate and the peptide molecular weights.

**Analysis of reaction kinetics of acrylate, crotonylate and dimethylacrylate-terminated PEGs with cysteine**

The amino acid cysteine was mixed in solution (0.1M phosphate, pH 8.0) with functionalized PEGs (PEG-8000-2A, PEG-8000-2C, and PEG-8000-2DMA) such that thiols and conjugated unsaturated groups were initially in equimolar concentrations (20 micromolar). In the presence of dimethacryloyl functionalities, the rate of thiol consumption was essentially zero over the time scale followed (10 minutes). In the presence of the less sterically hindered crotonoyl functionalities (one methyl substitution on the double bond), the rate of thiol consumption was increased. In the presence of the even less hindered acrylates, the concentration of thiols decreased more rapidly, but did not go to completion in the time course followed. See Figure 5.

In a similar experiment where the concentration of conjugated unsaturated groups was ten times that of the thiol groups, the consumption of thiols by acrylates was extremely rapid. The reaction was complete by the taking of the first sample at time 1 minute (data not shown).

**Example 5: Demonstration of hydrolysis of the bond formed between a cysteine-containing peptide and acrylated polymers**

**Hydrolysis in solution**

The peptide Ac-GCGYGRGDSP-NH<sub>2</sub> was dissolved in deionized water, and PEG-8000 diacrylate was dissolved in deionized water buffered with 10 mM HEPES and 115 mM triethanolamine at pH 8. After mixing 1 mol of the peptide per 2 mol of the PEG-8000 diacrylate, the reaction was followed by C18 chromatography, using a gradient from 95% water with 0.1% trifluoroacetic acid, 5% acetonitrile to 40% water with 0.1% trifluoroacetic acid, 60% acetonitrile. The peptide Ac-GCGYGRGDSP-NH<sub>2</sub>, eluted at about 20% acetonitrile, whereas PEG or PEG-8000 diacrylate eluted at about 40%

5 acetonitrile. Rapidly, the free peptide peak at 20% acetonitrile disappeared, and the  
peptide then coeluted with the PEG peak at 40% acetonitrile. The solution containing the  
PEG-peptide adduct was then incubated at 37°C, and C18 chromatographic injections  
10 were made at later time points to detect hydrolysis of the peptide from the polymer. This  
was measured by observing the decrease in signal at 273 nm that coeluted with the PEG  
peak, and the reappearance of the free peptide peak at about 20% acetonitrile. MALDI-  
15 TOF mass spectrometry of the new peak eluting at about 20% acetonitrile revealed a  
product of molecular weight which corresponded to the molecular weight of the original  
peptide plus 72 mass units. This indicated that the new peak contains peptide modified  
20 with propionic acid, which was the product that would be expected following conjugate  
addition between the cysteine on the peptide and an acrylate group, followed by  
hydrolysis of the ester of the modified acrylate. A half-life for hydrolysis of the ester  
between the peptide and the PEG was found to be 4.86 days. This corresponds to a half-  
25 life of hydrolysis of about 3 weeks at pH 7.4.

15 **Degradation of gels formed by reaction of polymers containing thiols and acrylates**

30 PEG-3400 triacrylate was dissolved in 50 mM HEPES buffered saline, pH 8 at a  
concentration of 20% (w/v). PEG-3400 dithiol (Shearwater Polymers, Huntsville, AL,  
USA) was dissolved in 1 mM MES buffered saline, pH 5.6 at a concentration of 20%  
35 (w/v). The solutions were mixed at a ratio of 1 acrylate : 1 thiol. Gels formed after a few  
20 minutes at 37°C, and the gels were then transferred to tubes containing 10 mM HEPES  
buffered saline at pH 7.4, and were incubated at 37°C. The HEPES buffered saline was  
40 replaced daily for the first week, and the presence of a gel remaining in the tube was  
assessed daily. Solid gels were found to be gone from the tubes after about 3 weeks, with  
solid gels absent from the tubes between 18 and 24 days after cross-linking. This is  
45 25 compared with gels formed from PEG-8000 diacrylate by free-radical cross-linking

5 (Pathak, supra), which are still present after 4 months at pH 7.4, 37°C.

**Degradation of gels formed by reaction of molecules containing thiols and acrylates**

10 13.9 mg PEG-2500-3A was dissolved in 69.5 microliters (5.0 mg/25 microliters)  
of PBS•TEA (10 mM PBS containing 13 microliters of triethanolamine/ml). 7.0 mg of  
5 GCYKNRDCG was dissolved in 65 microliters of PBS•TEA (2.7 mg/25 microliter).  
15 Equivolumes (25 microliters) of the two solutions were added to wells of a Corning flat-  
bottomed tissue culture treated polystyrene 96 well plates. As the second of the two  
precursor solutions was added, the pipet tip was used to stir the mixture for 2-3 seconds.  
20 Then the gels were allowed to set at 37°C. The gels were then transferred to tubes  
10 containing 10 mM HEPES buffered saline, pH 7.4. The gels were incubated at 37°C, and  
the disappearance of the solid gels was followed visually. Between 14 and 21 days, all of  
25 the solid gels were gone, indicating that they had degraded by hydrolysis of the ester bond  
between the peptide and the PEG.

**Control of the rate of hydrolysis via change in the local environment**

30 13.9 mg PEG-2500-3A is dissolved in 69.5 microliters (5.0 mg/25 microliters) of  
15 PBS•TEA (10 mM PBS containing 13 microliters of triethanolamine/ml). 7.0 mg of  
GKKKKGCYKNRDCG (SEQ ID NO: 65) is dissolved in 65 microliters of PBS•TEA  
35 (2.7 mg/25 microliter). Equivolumes (25 microliters) of the two solutions are added to  
wells of a Corning flat-bottomed tissue culture treated polystyrene 96 well plates. As the  
20 second of the two precursor solutions is added, the pipet tip is used to stir the mixture for  
2-3 seconds. Then the gels are allowed to set at 37°C. The gels are then transferred to  
40 tubes containing 10 mM HEPES buffered saline, pH 7.4. The gels are incubated at 37°C,  
and the disappearance of the solid gels is followed visually. The extra lysines found in  
45 the peptide ("GKKKK...") are added so as to provide additional nucleophiles to the local

5 environment of the ester bond. Additionally, the cationic nature of the groups may also  
lead to a raising of the local pH. The combination of these two effects is expected to  
enhance the rate of hydrolysis of the ester bond between the peptide and the polymer.

10  
15 **Example 6: Demonstration of plasmin hydrolysis of gels formed by conjugate  
addition with a peptide containing two cysteine residues with a plasmin substrate  
sequence in between, and lack of hydrolysis of a substituted peptide**

**Synthesis of gels by conjugate addition**

20 Since enzymes and peptides are chiral, the stereochemistry of GCYKNRDCG was  
altered to make a plasmin-stable nucleophile for gels made by conjugate addition. This  
10 plasmin stable peptide was: GCY-dLys-N-DArg-DCG (SEQ ID NO: 66). The sequence  
was otherwise not altered in order to maintain the extremely good water solubility  
properties of GCYKNRDCG.

25 Analytical C18 HPLC (linear acetonitrile gradient over 0.1% TFA in water) was  
used to confirm the relative plasmin-stability of GCY-dLys-N-DArg-DCG. The  
30 15 following samples were run: plasmin; GCYKNRDCG; plasmin + GCYKNRDCG; GCY-  
dLys-N-DArg-DCG; and plasmin + GCY-dLys-N-DArg-DCG. Plasmin (micromolar)  
was present at 1/1000 the concentration of the peptide (millimolar) and hence did not  
affect overlain absorbance chromatograms. Overlaying the traces (absorbance at 220 nm  
35 or 278 nm) of the peptide elutions vs. those of the peptide - plasmin, demonstrated that  
20 the most of the GCYKNRDCG peptide was degraded in approximately 1 hour at 37°C.  
The GCY-dLys-N-DArg-DCG peptide however, was unaffected by the plasmin at 24  
40 hours, and remained unaffected over the lifetime of the plasmin in the sample (sample  
injected for C18 at 2 weeks).

**Demonstration of plasmin-sensitivity and plasmin-resistance**

Gels were made according to the 40 microliter protocol given above. Some contained the GCYKNRDCG peptide with Lys and Arg in the L configuration. Another contained the GCY-DLys-N-DArg-DCG instead. All were exposed to 0.2 units of plasmin in 200 microliters and incubated at 37°C. The L-Lys, L-Arg configuration of the peptide was readily degraded by the enzyme. In one case, after 6 hours no gel remained. The DLys, DArg configuration gel has not been shown to degrade by plasminolysis.

**Example 7: Incorporation of peptides into polyethylene glycol gels formed by photopolymerization****Gel synthesis**

Polyethylene glycol diacrylate of mol. wt. 8000 (230 mg/ml) was allowed to dissolve in HEPES buffered saline (10 mmol HEPES, Sigma, 8 g/L NaCl, pH 7.4) for 1 hr. Triethanolamine (Aldrich, 15.3 µl/ml) was added, and the pH of the solution was adjusted to pH 8 with 6 N HCl. Cysteine containing peptides were dissolved in 116.5 µl of HEPES buffered saline, and added to 870 µl of the PEG solution with vortexing. After 5 min, 3.5 µl of N-vinyl pyrrolidone and 10 µl of a 10 mM solution of Eosin Y were added, followed by vortexing. Gels were formed by exposure to light at 75 mW/cm<sup>2</sup> for 1 min (Cerman Xenon Lightsource, transmitting light between 470 and 520 nm; ILC Technology, Sunnyvale, CA, USA). Gels were allowed to swell in Tris buffered saline, pH 7.4 (4.36 g Tris HCl, 0.64 g Trizma base, 8 g NaCl, 0.2 g KCl per 1 L, all from Sigma) for 36 hr.

Cell interaction with PEG gels containing peptides incorporated by conjugate addition in which the gel is formed by photopolymerization

PEG gels were prepared as described above, using the peptide GCGYGRGDSPG. Most cells have receptors that recognize the sequence GRGDSPG, and cells will interact

5 with surfaces displaying immobilized RGD containing peptides. To test cellular  
interactions of cells with PEG gels containing peptides incorporated via conjugate  
addition, gels were formed and human umbilical vein endothelial cells were seeded onto  
10 the gels. The change in the shape of the cells on the surface was observed, which  
5 indicated that the cells were interacting with the peptides on the surface. The change in  
shape is referred to as spreading, and refers to the change of the cell shape from spherical  
15 to flattened and polygonal on the surface. No cell spreading occurred on the PEG gels  
without peptide, and the specificity of the GCGYGRGDSPG peptide was confirmed by  
comparison with gels containing the peptide GCGYGRDGSPG, which contains the same  
20 10 amino acids, but in a different sequence, and which has no biological activity. Cells were  
seeded onto the gels at a concentration of 400 cells per mm<sup>2</sup>, and the number of spread  
cells per area were counted at different times (see Figure 6). The experiments were  
25 performed using the normal cell culture medium. Cells could only spread on gels that  
contained the peptide GCGYGRGDSPG, which was incorporated into the gels utilizing a  
15 conjugate addition reaction.

30 **Example 8: Formation of pH sensitive gels using conjugate addition reactions**

The peptide GCCGHHHHHGCCG (SEQ ID NO: 67) was synthesized as  
described above. The peptide (10 mg) was dissolved in 15 µl of 10 mM phosphate  
35 buffered saline, pH 7.4 and 25 µl ethanol. The pH of the solution was adjusted to pH 5.8  
using 1N NaOH, and then PEG diacrylate, mol. wt. 400 (7.5 µl, Aldrich), was added.  
20 The mixture was incubated at 37°C for 5 min. A hydrogel was formed, that demonstrated  
40 about a 50% increase in diameter upon changing from pH 7.4 to pH 5.8.

Gels were polymerized as spheres by adding the gelling solution from above to 1  
ml of cyclohexane containing 94 mg of Hypermer B239 (ICI Surfactants, Wilmington,  
45 25 DE, USA), with vortexing at 37°C for 10 min. Spheres were produced with diameters

5 ranging from 2  $\mu\text{m}$  to 20  $\mu\text{m}$ , which demonstrated about a 50% increase in diameter upon  
changing from pH 7.4 to pH 5.5.

10 **Example 9: Formation of particles for protein delivery applications**

PEG-3400 triacrylate is dissolved in 50 mM HEPES buffered saline, pH 8 at a  
5 concentration of 20% (w/v), with 2% albumin (Sigma, St. Louis, MO, USA). PEG-3400  
15 dithiol (Shearwater Polymers, Huntsville, AL, USA) is dissolved in 1 mM MES buffered  
saline, pH 5.6 at a concentration of 20% (w/v). The solutions are mixed at a ratio of 1  
acrylate : 1 thiol. The liquid solution (50  $\mu\text{l}$ ) is rapidly added to 1 ml of cyclohexane  
20 containing 100 mg Hypermer B239 (ICI Surfactants, Wilmington, DE, USA), with rapid  
10 stirring. The mixture is heated to 37°C for 30 min. The polymerized, protein-containing  
spheres are then washed with additional cyclohexane to remove surfactant, followed by  
25 drying in vacuum to remove cyclohexane. The particles are then resuspended in HEPES  
buffered saline, pH 7.4. Release of protein from the microspheres is measured by  
changing the resuspending medium daily, and protein in the resuspending medium is  
15 assessed using size exclusion chromatography combined with UV detection at 280 nm.  
30 Protein concentrations in the resuspending medium are determined from a concentration  
standard curve for albumin at 280 nm.

35 **Example 10: Targeting PEG-triacrylate microspheres to cells and tissues using  
peptides incorporated via conjugate addition**

20 Microspheres are formed via conjugate addition cross-linking of PEG-triacrylate  
40 and the peptide GCYdKNdRDCG (SEQ ID NO: 68) as in Example 7, but additionally the  
peptide GCGYGRGDSPG is also included in the reaction mixture, at a ratio of 1  
GCGYGRGDSPG to 8 GCYdKNdRDCG. The bioactive peptide is tested for the ability  
45 to localize microspheres to the surfaces of cells, as compared with microspheres



5 containing no bioactive peptide.

**Example 11: Drug encapsulation and delivery by gels made by conjugate addition**

10 Because the conditions for forming the PEG gels by conjugate addition are quite mild (room temperature to 37°C, pH approximately 8.0, in aqueous solvent), drugs such as protein drugs are incorporated into the gels for delivery. Such mild conditions do not  
5 denature most proteins. The drug is incorporated in a number of fashions. In one method, protein or other drug (soluble in water, ethanol, acetonitrile or other solvent for both the PEG and the enzyme sensitive peptide and which can be exchanged for aqueous  
15 buffer) is entrapped in the pore spaces of the gel during gel formation. Because free cysteines are relatively rare in natural proteins, one need only worry in the minority of cases that the protein will be cross-linked to the gel. Also, selectivity of the reaction is quite good since the addition of conjugated unsaturated compounds to other nucleophiles  
20 in proteins (hydroxyls and amines) is extremely slow compared to sulfhydryls. When the drug is larger than the pore spaces of the gel in its swollen state, as controlled by the molecular weight and concentrations of the precursors, then the drug does not diffuse out  
10 of the gel at an appreciable rate but is rather released by surface enzymatic degradation of the gel.

35 **Diffusive and degradative control of protein release: Myoglobin release following entrapment in gel pore spaces**

20 The protein myoglobin (17,000 Da) was released from hydrogels made by conjugate addition between thiols and acrylates. PEG-3500-3A at 0.2 g/ml in PBS, pH 7.4 was mixed with a solution of the plasmin sensitive peptide GCYKNRDCG such that the concentration of thiols and acrylates was the same and the final concentration of PEG-  
40 3500-3A was 10% (precursor solution). To some of the precursor solution, myoglobin

5 was added (5.2  $\mu$ l of 9.8 mg/ml myoglobin solution per 195  $\mu$ l of precursor solution).  
Myoglobin was chosen as a model protein for growth factors, such as BMP-2, because of  
its similar size. 200  $\mu$ l aliquots of precursor solution with and without myoglobin were  
10 made onto hemostatic collagen sponges. To some control sponges 5.2  $\mu$ l of the 9.8  
5 mg/ml myoglobin solution were added without gel precursors. To some sponges, PBS  
was added instead of myoglobin. After gels had solidified within the sponges, each  
15 sample was incubated in 4 ml of 10 mM PBS, pH 7.4, containing 0.1% sodium azide to  
prevent bacterial and fungal contamination. At 6 hr, 12 hr, 24 hr, 2 d, 3 d, 7 d, and 13 d  
the solution phase was removed from each sample and replaced with fresh PBS with  
20 10 0.1% sodium azide. After day 13, the solutions were replaced with 0.08 units of plasmin  
in 4 ml PBS, the discontinuity marked by the vertical line in Figure 7. Solutions were  
developed using the BIORAD/Bradford protein microassay and compared to a standard  
25 curve made from myoglobin solutions of known concentration. The samples with  
myoglobin within the hydrogel material showed a delayed release of the myoglobin  
15 (diffusion limited) but did, following hydrogel degradation by the enzyme plasmin,  
release a total amount of protein not different from the total released from the sponges  
30 alone (no hydrogel) (data not shown).

#### Release employing incorporated affinity sites

35 In another method, drugs such as heparin binding growth factors are  
20 electrostatically sequestered within the gel. This method is effective for trapping  
relatively low molecular weight compounds that otherwise diffuse out of the gel through  
40 its pores, especially in the swollen state of the gel. The sequestering is accomplished in a  
variety of methods.

In a first approach, one includes during gel formation by conjugate addition a  
45 25 heparin-binding peptide that contains one or more cysteines (i.e., the peptide can be

5 pendant or serve as cross-linker), heparin, and the heparin-binding growth factor. In a  
second approach, one makes one's unnatural proteins with molecular biological  
10 techniques and engineers in heparin-binding regions where none (or where only low  
affinity ones) existed before. In a third approach, one makes unnatural proteins that  
5 contain unpaired cysteines. Then one covalently couples the protein via this cysteine  
linker to the gel during the gel formation process. In a fourth approach, one engineers  
15 both an unpaired cysteine and an enzyme-sensitive region into an unnatural protein. This  
protein is also covalently incorporated into the conjugate addition gels at gel formation,  
but this protein is released in the presence of the proper protease, which can be the same  
20 10 that degrades the bulk of the gel or a distinct enzyme. In a fifth case one makes a heparin  
mimic containing a thiol, for example, a cysteine residue, or a conjugated unsaturation and  
covalently incorporates it into the material in the presence of a heparin-binding growth  
25 factor such that the growth factor is electrostatically sequestered.

**Incorporation of growth factor affinity and sequestering growth factors for  
15 prolonged release**

30 Heparin-binding proteins including heparin-binding growth factors are non-  
covalently bound to the material at material formation. If the protein to be bound does  
not contain a native heparin-binding sequence, a fusion protein is constructed (using  
35 molecular biological techniques and starting from the DNA level) to contain the native  
protein sequence and a synthetic heparin-binding domain.  
20

40 For example, nerve growth factor (NGF) is expressed as a fusion protein in *E. coli*  
such that the protein contains a heparin-binding domain at the N-terminus and the NGF  
sequence at the C-terminus of the protein. This is accomplished by constructing a  
synthetic gene containing the DNA which codes for the desired fusion protein. The  
45 25 protein sequence to expressed is as follows:

MGSSHHHHHHSSGLVPRGSHMKDPKRLYRSRKL PVELESSSHPIFHRGEFSVCDS  
VSVWVGDKTT.ATDIKGKEVMVLGEVNINNSVFKQYFFETKCRDPNPVDSGCRGI  
DSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRIDTACVCVLSRKA VRZ (SEQ ID  
NO: 69)

where the region in *italics* is the Histidine tag derived from the expression vector, and the  
underlined region is the thrombin cleavage site. Amino acids appearing in bold type  
denote the heparin-binding sequence.

The cloning plasmid used for gene assembly is pUC 18. The DNA sequence of the  
gene is as follows from 5' to 3':

GAATTCCCATGGCATATGAAAGACCCGAAACGTCTGTACCGTTCTCGTAAACT  
GCCCGTGGAAC TCGAGAGCTCTTCCCACCCGATTTTCCATCGTGGCGAGTTCT  
CCGTGTGTGACTCTGTCTCTGTATGGGTAGGCGATAAAACCACTGCCACTGAT  
ATCAAAGGCAAAGAGGTGATGGTGCTGGGAGAAAGTAAACATTAACAAC TCTG  
TATTCAAACAGTACTTCTTCGAAACTAAGTGCCCGTGACCCGAACCCGGTAGAC  
TCTGGGTGTCGCGGCATCGATTCTAAACACTGGAAC TCTTACTGCACCACTAC  
TCACACTTTCGTTAAAGCGTTGACTATGGATGGTAAACAGGCTGCCTGGCGTT  
TCATCCGTATCGATACTGCATGCGTGTGTGTACTGTCCCGTAAAGCTGTTTCGT  
TAAGGATCC (SEQ ID NO: 70)

This gene is inserted between the EcoRI and HindIII sites in the polylinker cloning region  
of pUC 18. After assembly, this gene is inserted into the expression vector. Expression  
and purification are then performed.

Using standard Fmoc peptide synthesis described above in Example 1, a heparin  
binding peptide, such as GCGK( $\beta$ A)FAKLAARLYRKA (SEQ ID NO: 71; see Table 5) is  
synthesized. For material formation, the peptide is preincubated with the conjugated  
unsaturated precursor; the fusion protein is preincubated with heparin; then the fusion

5 protein-heparin complex is added to the peptide-unsaturation such that the peptide is  
covalently coupled to the conjugated unsaturation and simultaneously heparin forms a  
10 non-covalent bridge between the peptide and the protein. Then the thiol containing cross-  
linking precursor is added, and the material is formed with the heparin-bound protein  
5 throughout.

### 15 Covalent incorporation of proteins and potential for enzymatically controlled release

A fusion protein is constructed to contain the protein of interest and at one  
terminus a short peptidyl sequence degradable by an enzyme, such as plasmin, and a  
20 cysteine distal to the site for proteolysis. The cysteine allows covalent incorporation of  
the protein in the material at material formation. The site for proteolysis allows for  
25 release of the protein in its native form at a rate determined by cellular activity, for  
example, activation of proteases such as plasmin or collagenase used in cell migration.  
The release of the protein can be controlled by the same or by a different enzyme than the  
15 one that degrades the material itself. There are also cases where covalent binding of the  
protein to the material without enzymatic release is desired. In these cases, the protein is  
30 engineered starting from the DNA level to contain an unpaired cysteine (e.g., at one of the  
termini of the protein) but no new site for proteolysis.

For example, the DNA for vascular endothelial growth factor (VEGF) is modified  
35 using site directed mutagenesis to introduce a cysteine near the N terminus of the protein.  
Molecular biological techniques are used to synthesize, purify and fold the protein. The  
20 protein is incubated with PEG-triacrylate with acrylates in excess of thiols in the protein.  
A plasmin sensitive peptide containing two thiols (GCYKNRDCG) is added to cross-link  
40 the material with the growth factor incorporated throughout.

**Example 12: Drug (including non-protein drug) delivery from a material via covalently linked prodrugs which can be released as drugs by proteolysis**

Another method for the covalent incorporation of prodrugs within a material is described. One can deliver a prodrug from a material where the material is soluble. Large molecular weight modifications of drugs are used to modulate circulation time, prodrug targeting, immune tolerance and mode of cellular uptake. A stable carrier-drug linkage is desired for transport. However, a bond that can be cleaved upon arrival at the desired location is of interest. An amide bond where one constituent of the bond is an L-amino acid recognized by a proteolytic enzyme is appropriate. Kopecek et al. (Pato, J., M. Azori, K. Ulbrich, and J. Kopecek, Makromol. Chem. 185: 231-237, 1984.) have published a great deal with regard to such enzymatically degradable soluble macromolecular drug carriers. However, little work appears with regard to enzymatically controlled drug delivery from solid materials, such as hydrogels. Delivery from a solid material serves to localize drug delivery to a desired site, for example, the site of material formation. Delivery from a solid material where the drug release is performed by cellular activity, such as expression of proteolytic enzymes, controls the rate of release such that cellular activity (e.g., cell migration) determines the rate of release.

The functional groups of a drug (such as anti-cancer drugs doxorubicin or daunorubicin) are protected with the exception of amine functional groups. The amine groups on the drug are coupled to an amino acid or peptide by formation of an amide bond. The amino acid or peptide is chosen to be degradable amino-terminal to the amino acid (Y) or peptide (XXXXY; SEQ ID NO:72), hence at the amide bond that joins the amino acid or peptide to the drug, by a proteolytic enzyme, such as plasmin which cleaves amino-terminal to lysine and arginine. Either a thiol (e.g., cysteine) is included in the coupled peptide or is next coupled to the amino acid or peptide portion of the peptide-

5 drug conjugate. The drug and peptide functional groups are deprotected (to give SH-XXXXY-drug). At material formation, the thiol-peptide-drug conjugate is covalently  
10 coupled to the material by way of conjugate addition of the thiol on a conjugated unsaturation in the material precursor. The drug is released from the material by  
5 enzymatic activity, such as plasminolysis, that cleaves the amide bond (Y-drug) linking the drug to the material.

15 Alternatively, the functional groups of a drug (such as the prostaglandin antagonist diclofenac) are protected with the exception of carboxyl functional groups. The carboxyl  
groups are activated and coupled to a peptide by formation of an amide bond at the  
20 10 peptide amino terminus. The peptide is designed to contain a thiol (e.g., cysteine) and to be degradable carboxyl-terminal to the peptide, hence at the amide bond that joins the  
peptide to the drug, by a proteolytic enzyme which cleaves carboxyl-terminal to specific  
25 amino acids (Y). The drug and peptide functional groups are deprotected. At material  
formation, the thiol-peptide-drug conjugate (drug-YXXXX-SH) is covalently coupled to  
15 the material by way of conjugate addition of the thiol on a conjugated unsaturation in the  
material precursor. The drug is released from the material by enzymatic activity that  
30 cleaves the amide bond (drug-Y) linking the drug to the material.

### Example 13: Tissue Regeneration

#### **Ectopic (subcutaneous) bone formation in the rat**

35 20 Materials were made essentially according to Example 3, but under sterile  
conditions and with PEG-3500-3A, a molar ratio of acrylates: thiols of 1:1, and a molar  
40 ratio of GCGYGRGDSPG: acrylates of 1/12. At the time of gel formation, a recombinant  
human growth factor, BMP-2, which induces bone formation was added to the precursor  
solution at a concentration of 250 µg/ml of precursor solution. Precursor solution was  
45 25 added to hemostatic collagen sponges (Helistat; 8 mm diameter, approximately 3.5 mm

5 height). Precursor solution was added until the sponges could not absorb more solution  
(approximately 160  $\mu$ l). The gels were allowed to solidify in the sponges. Gels were  
10 briefly washed with PBS then kept minimally wet until implantation subcutaneously in  
rats. The implants were removed after two weeks, fixed, and hematoxylin and eosin  
15 stained. The materials were well infiltrated by cells with very little residual material  
remaining and promoted bone formation (mineralization and marrow formation) and  
vascularization. This indicates that the materials can deliver active biomolecules (e.g.,  
growth factors) and can be infiltrated by cells *in vivo*.

#### 20 Bone regeneration

10 Hydrogel materials can be useful in bone regeneration in a variety of healing  
situations, for example, following trauma, tumor removal, or spinal fusion. In one  
example, hydrogel material, for example, as described above, is applied in the space  
25 within a spinal fusion cage, containing within that material a bone morphogenetic protein,  
such as BMP-2, TGF- $\beta$ , or BMP-7. This bioactive formulation is useful in enhancing the  
probability of successful spinal fusion within the case. Use of such a material can  
30 circumvent some of the difficulties with current surgical methods, such as filling the  
space within the cage with amorphous bone allograft (associated with disease  
transmission and high cost) and filling the space with bone autograph, for example,  
35 obtained from the iliac crest (associated with additional morbidity and hospitalization  
cost).

#### 40 Skin regeneration

Hydrogel material can be useful in skin healing and regeneration, for example, in  
the closure of diabetic foot ulcers, pressure sores, and venous insufficiency ulcers. A  
45 hydrogel, for example, as described above, can be used to delivery growth factors that



enhance the closure of these wounds, such as vascular endothelial cell growth factor, a TGF $\beta$ , activin, keratinocyte growth factor, platelet-derived growth factor, epidermal growth factor, or a number of other growth factors. These growth factors may be incorporated within the hydrogel either by entrapment or by biospecific affinity (e.g., by affinity for heparin).

**Example 14: Hydrogel and Non-hydrogel Materials for Bearing Structural Loads**  
**Structural materials formed by conjugate addition reactions**

Pentaerythritol tetrakis (3-mercaptopropionate)(QT) (424 mg) and 997 mg of polyethylene glycol diacrylate 570 (PEGDA) were combined neat and mixed well by vortexing. Air bubbles were removed by sonicating. PBS 0.01 M solution prepared at pH 9.0 (10 mM PBS adjusted to pH 9.0 with triethanolamine (EtOH<sub>3</sub>N) mixed with an equal volume of 10 mM PBS adjusted to pH 9.0 with 1N NaOH) (473 mg) was added to the mixed precursors. The mixture was again vortexed for about 2 minutes to mix well and disperse the precursors in the aqueous solution. Following vortexing, the mixture was again sonicated to remove air bubbles. At room temperature the material gelled in about 20 to 30 minutes. The resulting gel demonstrated ultimate strength of about 2 MPa and withstood deformations of about 35% in compression (Figure 8).

**Control of mechanical properties by hydrophobicity (Pentaerythritol triacrylate)**

QT and pentaerythritol triacrylate were mixed neat at a ratio of 489 mg to 596 mg (mixture 1). QT and PEGDA 570 were mixed at the ratio indicated above (mixture 2). 100 mg of mixture 1 was combined with 650 mg of solution 2 and 250 mg of PBS pH 9.0 was added and the entire mixture was vortexed to mix. Similar gels were prepared for 200, 300, and 400 mg of mixture 1. To these were added 550, 450 and 350 mg of mixture 2 respectively. To all of these 250 mg of the activating buffer was added. The resulting

gels demonstrate a modulation of the mechanical properties using the addition of a hydrophobic coprecursors. An increase in the content of the hydrophobic TA increased the stiffness of the resulting gel (Figure 9).

#### Varying thiols to acrylate ratio

QT and PEGDA 570 were combined neat to achieve ratios of thiol to acrylate of 0.8, 0.9, 1.0, 1.1, 1.3 by adding the appropriate amount of QT to 1000 mg of PEGDA 570 and adding PBS 9.0 in a quantity to make 75 wt% solid gels. As an example, for the 0.8 thiol to acrylate ratio, 343 mg of QT were added to 1000 mg of PEGDA. The two components were mixed by vortexing and then 448 mg of PBS 9.0 was added. Again the mixture was vortexed and then allow to gel. At thiol acrylate ratios from 1, the resulting gels exhibit significant decreases in ultimate strength. At ratios from 1.0 to 1.3, the gels were less sensitive to changes in the thiol/acrylate ratio. The table below (Table 8) presents the ultimate strengths obtain at each of the thiol/acrylate ratios.

Table 8: Ultimate strength of gels with various thiol/acrylate ratios

| Thiol/acrylate | Ultimate Strength |
|----------------|-------------------|
| .8             | $0.89 \pm 0.79$   |
| .9             | $0.64 \pm 0.07$   |
| 1.0            | $2.21 \pm 0.12$   |
| 1.1            | $2.29 \pm 0.13$   |
| 1.2            | $1.93 \pm 0.24$   |
| 1.3            | $1.82 \pm 0.23$   |

**Control of mechanical properties by addition of particles**

Precursors, QT and PEGDA 570, were combined as outlined above. Prior to addition of the activating buffer (pH 9.0 PBS), 10 wt% of BaSO<sub>4</sub> particles, balance fixed (0.8  $\mu$ m), were added to the mixed precursors. The activating buffer was added and then the entire mixtures were vortexed and then allowed to gel. The same quantities of the precursors, as noted in the example above, were used. The gels resulting from addition of the BaSO<sub>4</sub> showed some increase in ultimate strength and substantial increase in stiffness (Figures 10 and 11). QT and PEGDA 570 gels were also prepared that were loaded with fumed silica particles (14 nm). 424 mg of QT was combined with 997 mg of PEGDA. Prior to addition of the PBS pH 9.0 buffer, the buffer was loaded with 10% fumed silica. 250 mg of the PBS-fumed silica mixture was added to the QT/PEGDA mixture and then vortexed to mix. The gels resulting from the addition of fumed silica showed significant increases in the ultimate strength. Figure 12 presents the stress strain curve for the fumed silica gels in sub failure compression. At 4 MPa in compression these gels had not failed.

**Improvement of mechanical properties by use of emulsifiers**

Gels were prepared by emulsion by adding sorbitan monooleate to the PBS 9.0 buffer at 4 wt% prior to addition of the buffer to the mixed precursors. QT and PEGDA 570. The surfactant/pH 9.0 buffer mixture was then added to the mixed precursors. Otherwise the same quantities and procedures were used as noted above. The resulting gels exhibited a similar increase in ultimate strength compared to the gels with inorganic particles added but without the associated increase in stiffness (Figures 10 and 11).

**Preparations of materials in solvents including organic solvents**

QT and PEGDA were combined in the ratios indicated above. These precursors were then dissolved at 10 wt% in N-methyl pyrrolidinone (NMP) and then allowed to gel. After the gels cured for 24 hours, they were placed in deionized water to allow solvent exchange. During solvent exchange the volume of the gels reduced 60% to a new equilibrium volume. The resulting equilibrated gels showed soft elastic response to compression at low loads and an increase in stiffness with high deformation in compression. Figure 13 shows a typical stress strain curve for this material.

**Modulation of mechanical properties by addition of hydrophilic additives**

QT and PEGDA 570 were combined in the ratios indicated above. Poly(vinyl pyrrolidone) (40,000 MW) (PVP) was dissolved in the PBS 9.0 buffer at 1, 7, and 13%. The same quantities of the precursor mixture and the buffer/PVP solution were combined as indicated above. The mixture was vortexed and allowed to gel. These gels demonstrated the manipulation of mechanical properties due to the addition of the hydrophilic additive. The addition of PVP increased the equilibrium swelling of the gel and an increase in the PVP content further increased the swelling. An addition of PVP also resulted in a weaker softer gel.

**Kinetics of QT and PEGDA 570 gelation**

QT and PEGDA were combined neat in the ratios indicated above. After mixing the QT/PEGDA 570 by vortexing, the buffer was not added but instead 100 microliters of the mixture was placed between 20 mm plates of a CVO 120 rheometer with a gap of 100  $\mu$ m at room temperature. The mixture was maintained at room temperature while the elastic modulus, complex modulus and viscosity were followed with time using shear at 1 Hz with a strain amplitude of 0.3. With progression of the reaction the two combined

precursors showed a gel point, defined by the time when the elastic modulus becomes greater than the complex modulus, of about 14 hours. Figure 14 shows these two moduli for the combined precursors with time.

Next, more of the two precursors were combined as described above and the PBS 9.0 buffer was added, as described above. After mixing the precursors and buffer, the mixture was placed between the plates of the rheometer at 37°C. The frequency and amplitude were the same as the previous procedure. With the addition of the buffer, the kinetics of the gelation increased dramatically. At 37°C the gel point occurred in about 11 minutes. Figure 15 presents the moduli for the precursors activated with pH 9.0 buffer.

#### Biocompatibility of gels in tissue sites

Precursors, QT and PEGDA 570, the buffers, and sorbitan monooleate were all filter sterilized. Blanc Fixe particles were sterilized by autoclave. Gel pins were prepared using the precursors, Blanc Fixe and sorbitan monooleate. Other pins were prepared using only the two precursors. The gel pins prepared with only the two precursors were prepared using the same procedure cited above, except that the activated and vortexed mixture was placed in molds to form pins prior to gelation and the gel pins containing the inorganic particle and the surfactant were prepared by combining the procedures described above. The pins were implanted into the right and left dorsal muscles of rabbits. Reference pins of polyethylene were also used. After 4 weeks histological sections of the implants and the surrounding tissue were performed. With both gel types tested, no significant differences were apparent compared to the reference materials. Rare macrophages, fibroblasts and neovessels were associated with the implanted gel pins. No necrosis, degeneration or any other local intolerance signs were induced by these gel compositions.

5 Toxicity and biocompatibility of the low molecular weight precursors can be improved by pre-reacting the precursors at quantities that result in higher molecular weight precursors with remaining functional groups. QT has been functionalized with 10 fold excess PEG-DA 570. The result of this process is a tetrafunctional acrylate consisting of each thiol of the QT capped with a diacrylate leaving a terminal acrylate free. A similar reaction with QT in excess gives a peg capped at each end with three free thiols giving a hexafunctional thiol. The combination of these precursors with 1:1 thiol to acrylate ratio gives similar gels as obtained by the direct application of QT and PEGDA 570 (refer Figures 10 and 11, noting the HT and QA values).

#### 10 Control of mechanical properties by preparation of materials from precursors of mixed molecular weight

25 End functional polymer cross-linked systems have shown that mechanical properties can be manipulated by using multimodal molecular weight distributions. Including a low molar content of a high molecular weight precursor in a low molecular weight system has a synergistic effect giving improved mechanical properties than are achievable by either molecular weight alone. Networks containing only short chains are brittle and networks containing only the larger component have very low ultimate strength. While, bimodal systems having predominately the small chains with a small molar ratio of the larger component show networks with a high ultimate strength compared to the larger molecular weight system and with improved extensibility compared to the short chain monomodal system (Pathak, *supra*, Llorente, M. A., et al., J Polym. Sci., Polym. Phys. Ed., 19:621, 1981).

40 Low molar content of a larger molecular weight precursor (i.e., PEGDA 20,000 or PEVAL 20,000) can replace some of the PEGDA 570, creating a bimodal system. The three precursor system can be combined (i.e., QT, PEGDA 570 and PEGDA 20,000) in

an aqueous system at a pH providing sufficient reaction kinetics. The results are tougher gels. The hydrophilic/hydrophobic balance of this third (larger molecular weight precursor) can also be exploited to further modulate properties.

**Example 15: Preparation of Materials that are Responsive to Environmental Conditions**

**Temperature sensitivity of the precursors**

If the precursors are temperature sensitive (i.e., soluble below a critical temperature below 37°C and insoluble above the same temperature) but still possess thiol or conjugated unsaturated groups, they can be used to prepare gels with easy manipulation during mixing, but show the increased properties exhibited by gels obtained with the hydrophobic precursors. For this purpose either telechelic or grafted functional groups on poly(N-isopropylacrylamide), poly(propylene glycol-co-ethylene glycol), or other temperature sensitive polymers may be used. The precursors can be dissolved in water at a temperature below the critical temperature. The precursors solutions can be combined and allowed to gel. If the gel experiences a temperature increase above the critical temperature, then the gel will undergo a transition to a more hydrophobic state. The transition may or may not be associated with syneresis depending on the design of the temperature sensitive precursors and original concentrations.

**pH sensitivity of the precursors**

If the precursors are pH sensitive (i.e., soluble above or below a critical pH) but still possess thiol or conjugated unsaturated groups, they can be used to prepare gels with easy manipulation during mixing but show the increased properties exhibited by gels obtained with the hydrophobic precursors. For this purpose either telechelic or grafted functional groups on poly(N-isopropylacrylamide-co acrylic acid), poly(N-

5 isopropylacrylamide-co dimethylaminoethylmethacrylate) or poly(acrylic acid) or other  
pH sensitive polymers may be used. The solubility of these materials can be altered by  
pH. The precursors solutions can be combined and allowed to gel. A pH change in the  
10 environment changes the hydrophobicity of the gel by protonating or deprotonating the  
5 gel. The transition may or may not be associated with syneresis depending on the design  
of the pH sensitive precursors and original concentrations.

#### 15 Other Embodiments

From the foregoing description, it will be apparent that variations and  
20 modifications may be made to the invention described herein to adopt it to various usages  
10 and conditions. Such embodiments are also within the scope of the following claims. In  
addition, the use of an amine as a biomaterial precursor component is an embodiment  
25 which falls within the scope of the following claims.

All publications and patents mentioned in this specification are herein incorporated  
by reference to the same extent as if each individual publication or patent was specifically  
15 and individually indicated to be incorporated by reference.

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What is claimed is:



## Claims

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5 1. A method for making a biomaterial, said method comprising combining two or  
more precursor components of said biomaterial under conditions that allow  
polymerization of the two components, wherein said polymerization occurs through self  
10 selective reaction between a strong nucleophile and a conjugated unsaturated bond or a  
conjugated unsaturated group, by nucleophilic addition, wherein the functionality of each  
5 component is at least two, and wherein said biomaterial does not comprise unprocessed  
albumin, and said unsaturated bond or group is not a maleimide or a vinyl sulfone.  
15

20 2. The method of claim 1, wherein said components are selected from the group  
consisting of oligomers, polymers, biosynthetic proteins or peptides, naturally occurring  
10 peptides or proteins, processed naturally occurring peptides or proteins, and  
polysaccharides.  
25

30 3. The method of claim 2, wherein said components are functionalized to  
comprise a strong nucleophile or a conjugated unsaturated group or a conjugated  
unsaturated bond.  
35

15 4. The method of claim 1, wherein said strong nucleophile is selected from the  
group consisting of a thiol or a group containing a thiol.  
35

40 5. A method for making a biomaterial, said method comprising combining two or  
more precursor components of said biomaterial under conditions that allow  
polymerization of the two components, wherein said polymerization occurs through self  
20 selective reaction between an amine and a conjugated unsaturated bond or a conjugated  
unsaturated group, by nucleophilic addition, wherein the functionality of each component  
45 is at least two, and wherein said biomaterial does not comprise unprocessed albumin, and

5 said unsaturated bond or group is not a maleimide or a vinyl sulfone.

10 6. The method of claim 1, wherein said conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or 2- or 4-vinylpyridinium.

15 5 7. The method of claim 2, wherein said polymer is selected from the group consisting of poly(ethylene glycol), poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(acrylic acid), poly(ethylene-co-acrylic acid), poly(ethyloxazoline), poly(vinyl pyrrolidone), poly(ethylene-co-vinyl pyrrolidone), poly(maleic acid), poly(ethylene-co-maleic acid), poly(acrylamide), and poly(ethylene oxide)-co-poly(propylene oxide) block copolymers.

20 25 10 8. The method of claim 1, wherein one said component has a functionality of at least three.

30 9. The method of claim 2, wherein said peptide comprises an adhesion site, growth factor binding site, or protease binding site.

35 15 10. The method of claim 1, further comprising combining said precursor components with a molecule that comprises an adhesion site, a growth factor binding site, or a heparin binding site and also comprises either a strong nucleophile or a conjugated unsaturated bond or a conjugated unsaturated group.

40 45 20 11. The method of claim 10, wherein said strong nucleophile is a thiol or said conjugated unsaturated bond or conjugated unsaturated group is an acrylate, an acrylamide, a quinone, or a vinyl pyridinium.

5 12. The method of claim 1, wherein said biomaterial is a hydrogel.

10 13. The method of claim 1, wherein said biomaterial is degradable.

15 14. The method of claim 1, wherein said biomaterial is made in the presence of sensitive biological molecules.

20 15. The method of claim 1, wherein said biomaterial is made in the presence of cells or tissues.

25 16. The method of claim 1, wherein said biomaterial is made within or upon the body of an animal.

30 17. The method of claim 1, further comprising combining said precursor components with an accelerator prior to polymerization.

35 18. The method of claim 1, further comprising mixing said precursor components with a component that comprises at least one conjugated unsaturated bond or conjugated unsaturated group and at least one amine reactive group.

40 19. The method of claim 15, further comprising applying an additional component to the cell or tissue surface, the additional component comprising at least one conjugated unsaturated bond or conjugated unsaturated group and at least one amine reactive group.

45 20. A biomaterial formed by combining two or more precursor components of a biomaterial under conditions that allow polymerization of the two components, wherein

5 said polymerization occurs through self selective reaction between a strong nucleophile  
and a conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic  
addition, wherein the functionality of each component is at least two, said biomaterial  
10 does not comprise unprocessed albumin, and said unsaturated bond or group is not a  
5 maleimide or a vinyl sulfone.

15 21. The biomaterial of claim 20, wherein said component is selected from the  
group consisting of oligomers, polymers, biosynthetic proteins or peptides, naturally  
occurring peptides or proteins, processed naturally occurring peptides or proteins, and  
20 polysaccharides.

10 22. The biomaterial of claim 21, wherein said components are functionalized to  
25 comprise a strong nucleophile or a conjugated unsaturated bond or a conjugated  
unsaturated group.

30 23. The biomaterial of claim 20, wherein said strong nucleophile is selected from  
the group consisting of a thiol, or a group containing a thiol.

15 24. A biomaterial formed by combining two or more precursor components of a  
35 biomaterial under conditions that allow polymerization of the two components, wherein  
said polymerization occurs through self selective reaction between an amine and a  
conjugated unsaturated bond or a conjugated unsaturated group, by nucleophilic addition,  
40 wherein the functionality of each component is at least two, said biomaterial does not  
20 comprise unprocessed albumin, and said unsaturated bond or group is not a maleimide or  
a vinyl sulfone.

5 25. The biomaterial of claim 20, wherein said unsaturated group is an acrylate, an acrylamide, a quinone, or a vinylpyridinium.

10 26. The biomaterial of claim 21, wherein said polymer is selected from the group consisting of poly(ethylene glycol), poly(ethylene oxide), poly(vinyl alcohol),  
5 poly(ethylene-co-vinyl alcohol), poly(acrylic acid), poly(ethylene-co-acrylic acid),  
15 poly(ethyloxazoline), poly(vinyl pyrrolidone), poly(ethylene-co-vinyl pyrrolidone),  
poly(maleic acid), poly(ethylene-co-maleic acid), poly(acrylamide), and poly(ethylene  
oxide)-co-poly(propylene oxide) block copolymers.

20 27. The biomaterial of claim 20, wherein one said component has a functionality  
10 of at least three.

25 28. The biomaterial of claim 21, wherein said peptide comprises an adhesion site, growth factor binding site, or protease binding site.

30 29. The biomaterial of claim 20, further comprising a molecule that comprises an adhesion site, a growth factor binding site, or a heparin binding site and also comprises  
15 either a strong nucleophile or a conjugated unsaturated bond or a conjugated unsaturated  
35 group.

40 30. The method of claim 29, wherein said strong nucleophile is a thiol or said conjugated unsaturated bond or conjugated unsaturated group is an acrylate, a quinone, or a vinyl pyridinium.

45 20 31. The biomaterial of claim 20, wherein said biomaterial is a hydrogel.

5 32. The biomaterial of claim 20, wherein said biomaterial is degradable.

10 33. The biomaterial of claim 20, wherein said biomaterial is made in the presence of sensitive biological molecules.

15 34. The biomaterial of claim 20, wherein said biomaterial is made in the presence of cells or tissues.

20 35. The biomaterial of claim 20, wherein said biomaterial is made within or upon the body of an animal.

25 36. The biomaterial of claim 20, further comprising an accelerator.

30 37. The biomaterial of claim 20, further comprising a component that comprises at least one conjugated unsaturated bond or conjugated unsaturated group and a least one amine reactive group.

35 38. The biomaterial of claim 34, further comprising an additional component coupled to the cell or tissue surface, the additional component comprising at least one conjugated unsaturated bond or conjugated unsaturated group and being coupled to the  
15 cell or tissue surface via by reaction of at least one amine reactive group.

40 39. A method for delivering a therapeutic substance to a cell, tissue, organ, organ system, or body of an animal said method comprising the steps of contacting said cell, tissue, organ, organ system or body with the biomaterial of claim 20 or 24, wherein said  
45 biomaterial contains a therapeutic substance, whereby said therapeutic substance is

5 delivered to said cell, tissue, organ, organ system, or body of an animal.

10 40. The method of claim 39, wherein said therapeutic substance is selected from the group consisting of proteins, naturally occurring or synthetic organic molecules, viral particles, and nucleic acid molecules.

15 5 41. The method of claim 39, wherein said therapeutic substance is a prodrug.

42. The method of claim 40, wherein said nucleic acid molecule is DNA or RNA.

20 43. The method of claim 40, wherein said nucleic acid molecule is an antisense nucleic acid molecule.

25 44. A method of regenerating a tissue, said method comprising introducing a scaffold to a site, under conditions which permit cell ingrowth, said scaffold comprising the biomaterial of claim 20 or 24.

30 45. The method of claim 44, wherein said scaffold has been pre-seeded with cells.

35 46. The method of claim 44, wherein said tissue is selected from the group consisting of bone, skin, nerve, blood vessel, and cartilage.

40 15 47. A method of preventing adhesions, thrombosis, or restenosis, said method comprising the steps of contacting a site with the biomaterial precursor components of claim 20 or 24; and polymerizing said components at said site.



5 48. A method of sealing a fluid or gas flow, said method comprising the steps of  
10 contacting a site within the body of an animal with the biomaterial precursor components  
of claim 20, 24, or 37; and polymerizing said components at said site.

15 49. The method of claim 48, wherein said site is a lung, blood vessel, skin, dura  
5 barrier, and intestine.

20 50. A method of encapsulating a cell or tissue, said method comprising the steps  
of combining the precursor components of a biomaterial with a cell or tissue; and  
polymerizing said components, wherein said polymerization occurs through self selected  
10 reaction between a strong nucleophile and a conjugated or a conjugate unsaturated group,  
unsaturated bond, and wherein said cell or tissue is encapsulated by said polymerized  
25 biomaterial.

Figure 1

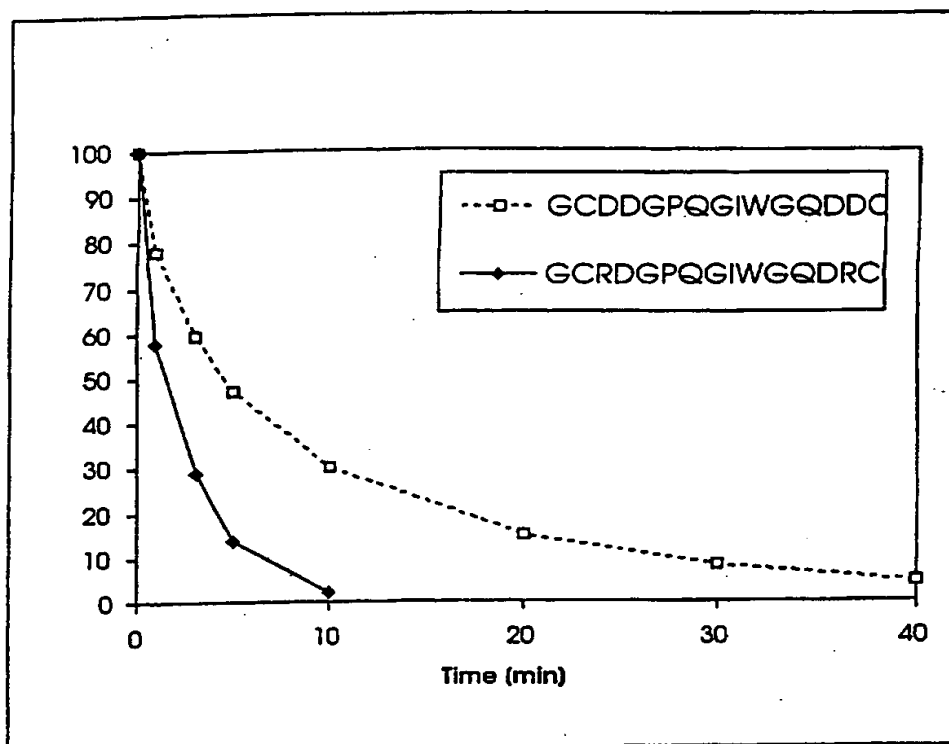


Figure 2

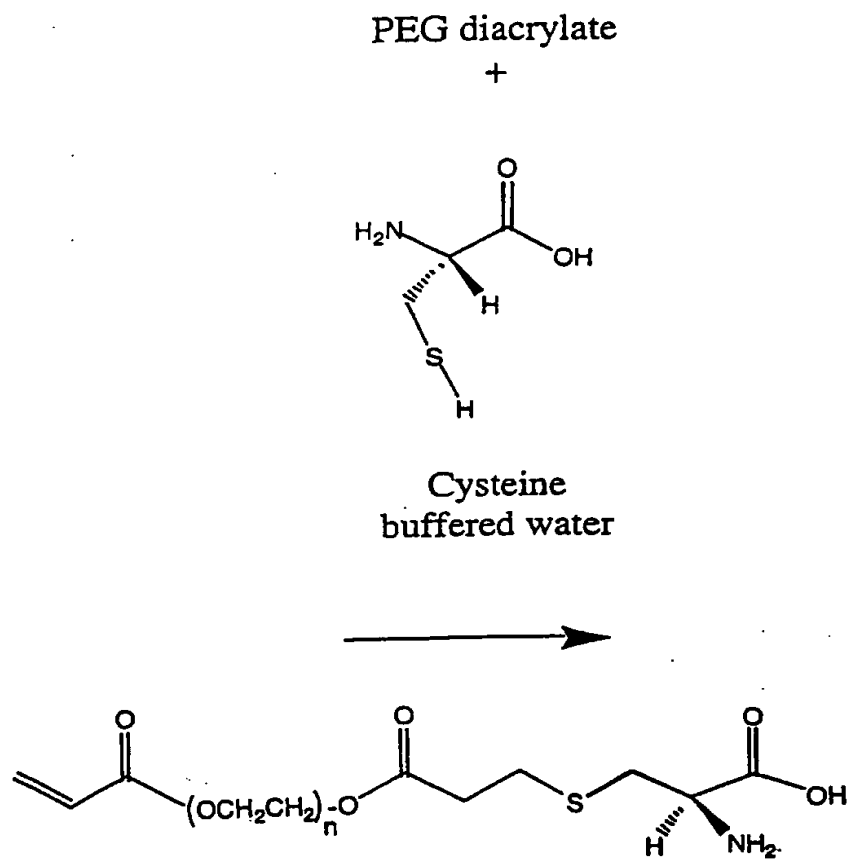


Figure 3

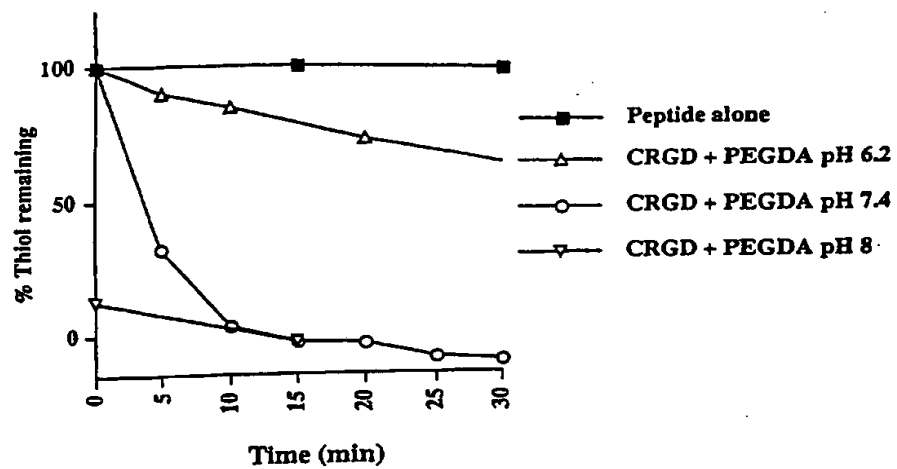


Figure 4

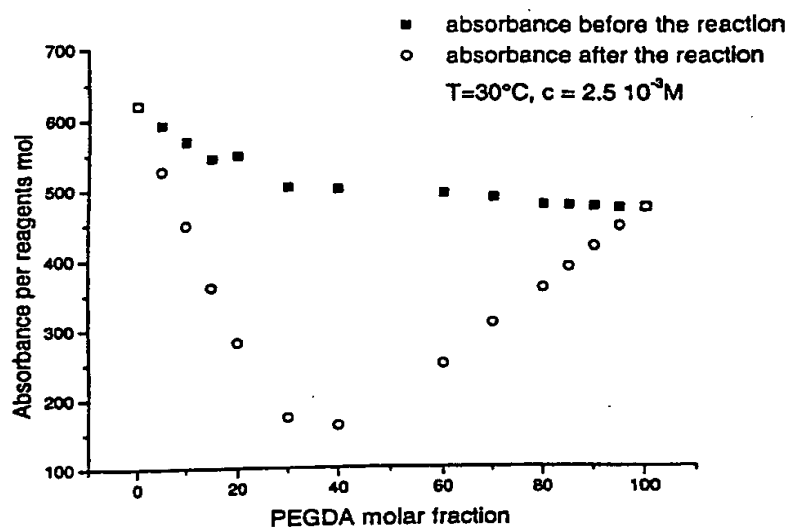


Figure 5

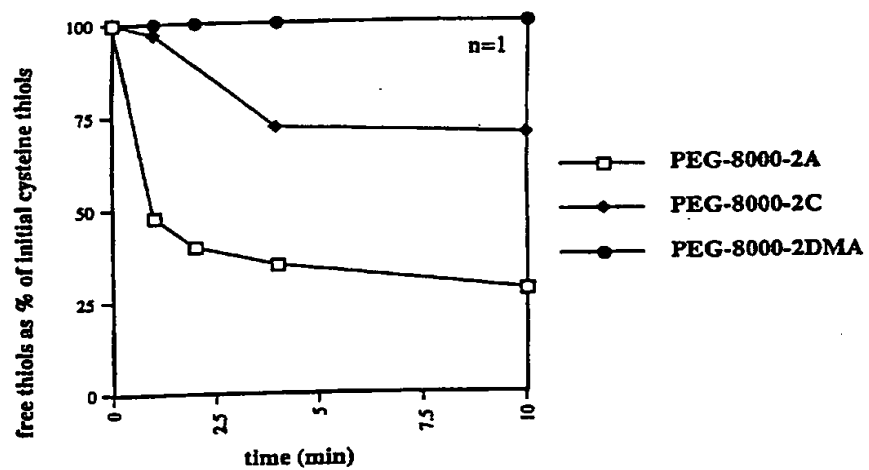


Figure 6

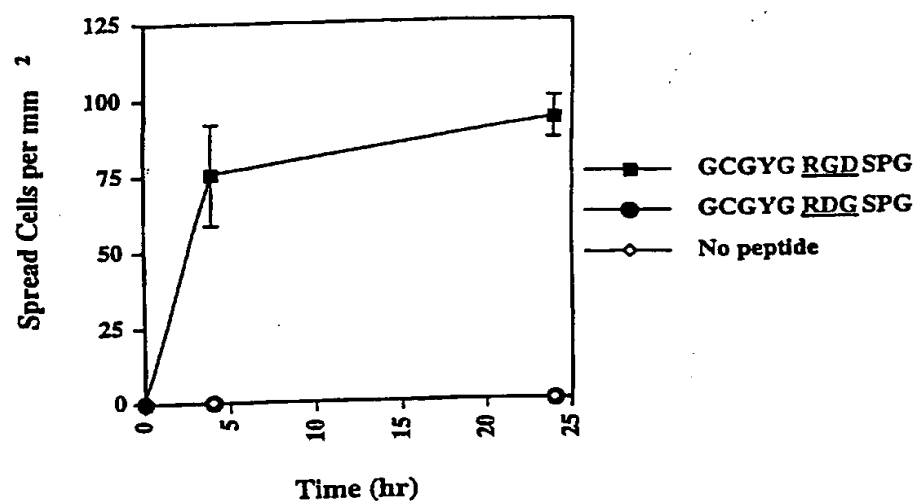


Figure 7

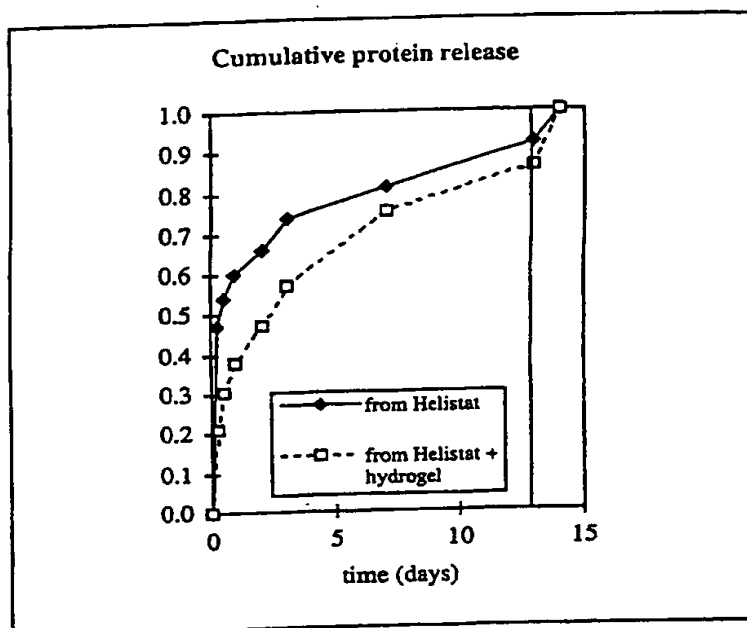




Figure 8

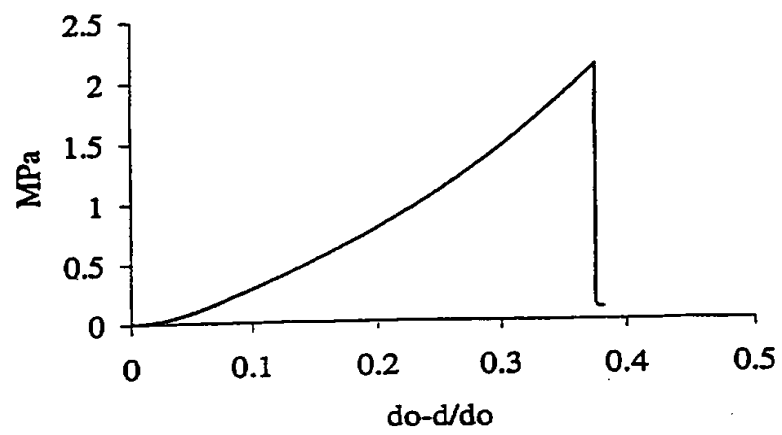


Figure 9

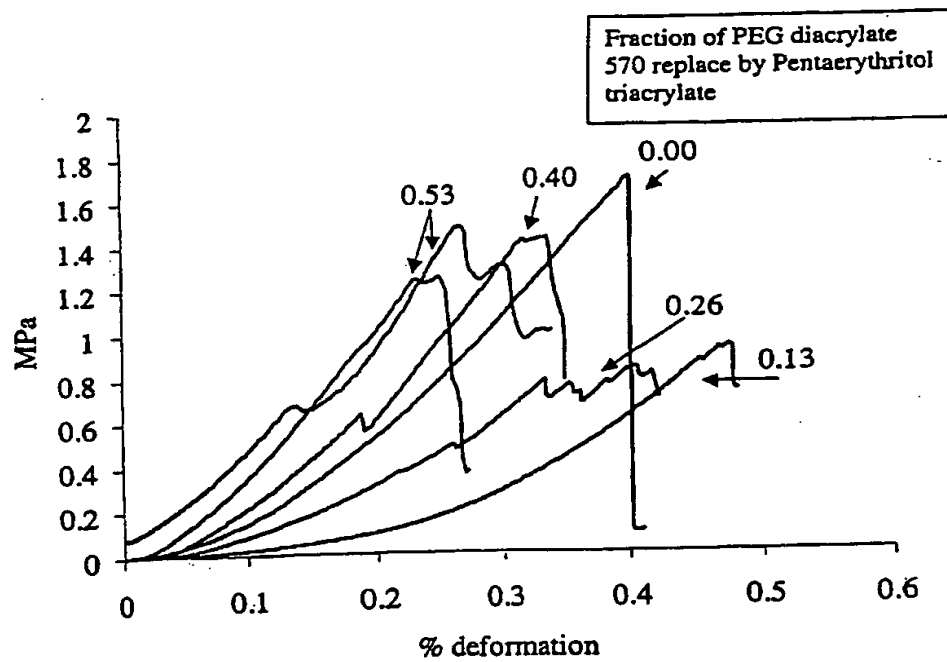


Figure 10

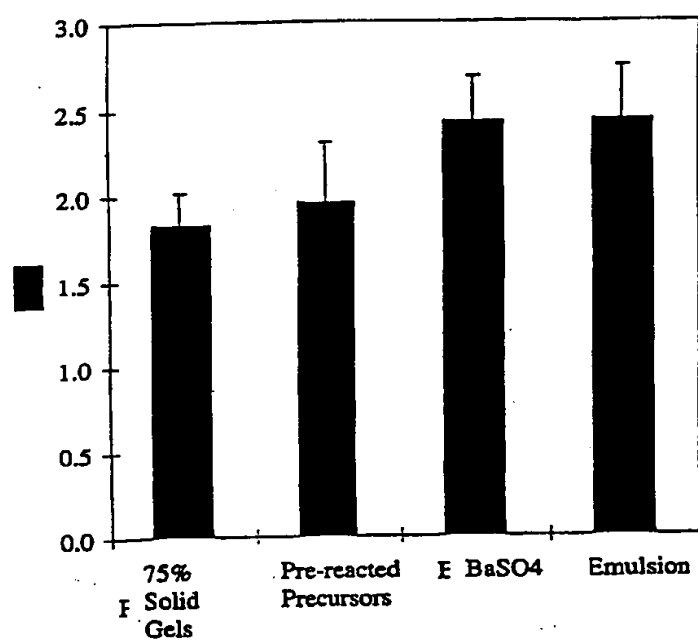


Figure 11

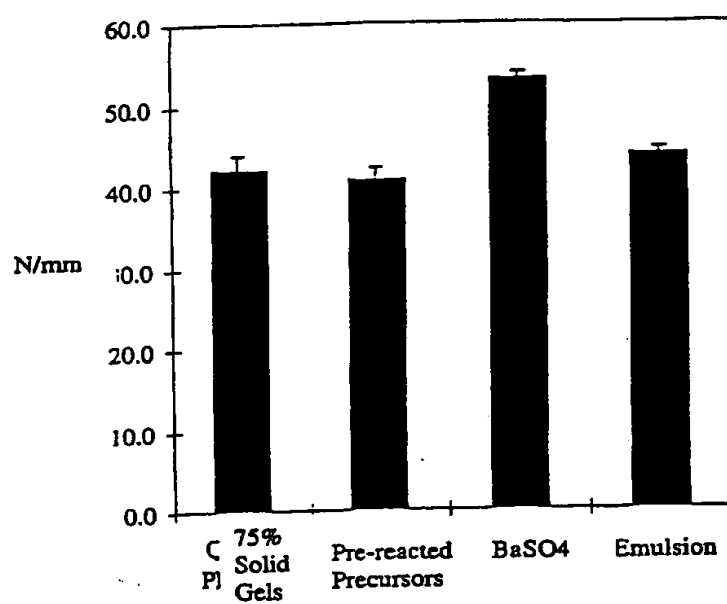


Figure 12

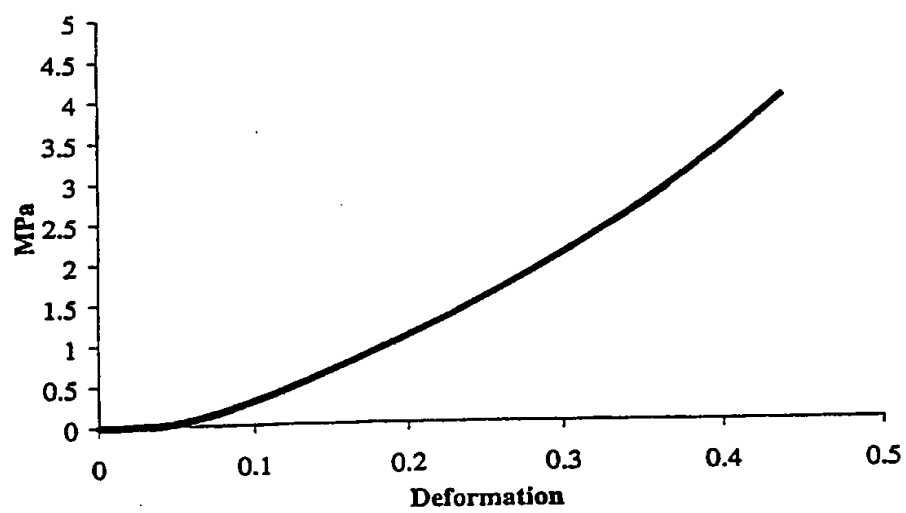


Figure 13

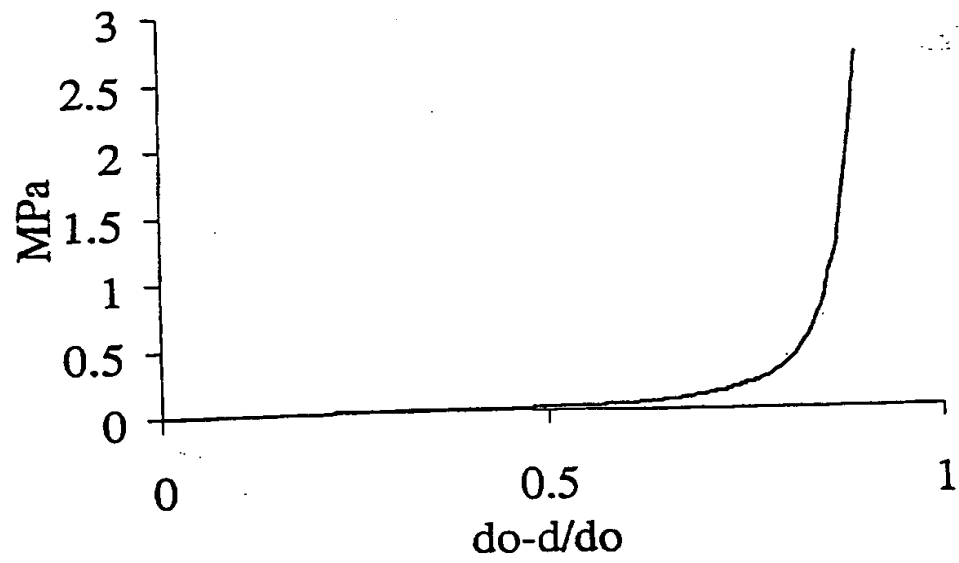


Figure 14

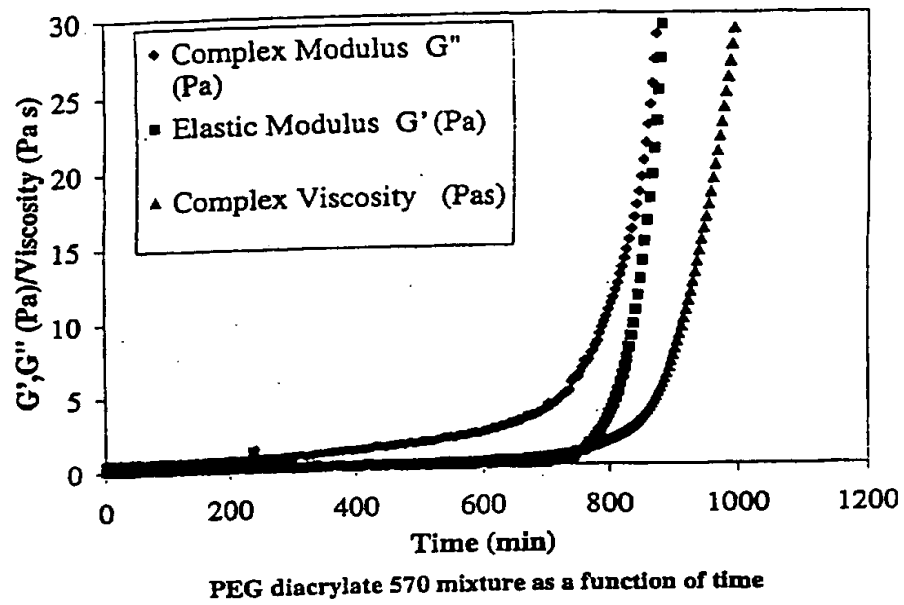
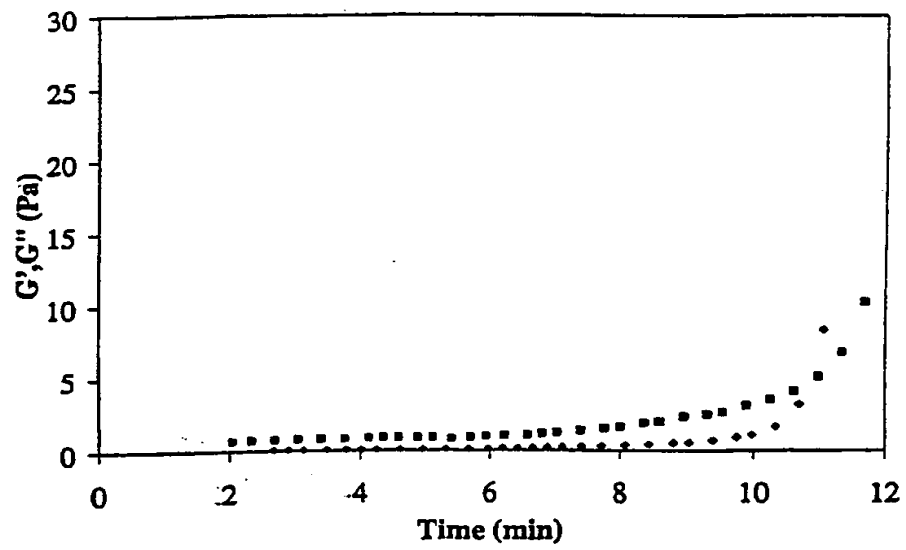


Figure 15





# INTERNATIONAL SEARCH REPORT

1. national application No.  
PCT/US00/02608

| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC(7) : Please See Extra Sheet.<br>US CL : Please See Extra Sheet.<br>According to International Patent Classification (IPC) or to both national classification and IPC  |  |  |
|---|--|--|
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>U.S. : 525/54.1; 530/812, 815, 816, 817; 435/177, 180, 181, 182; 436/531, 532, 533, 534, 535<br>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched<br>NONE<br>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>NONE   |  |  |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>   |  |  |
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No.  |
| Y   | US 5,268,305 A (RIBI et al) 07 December 1993, see entire document.                 | 1-50   |
| Y   | US 5,427,915 A (RIBI et al) 27 June 1995, see entire document.                     | 1-50   |
| Y   | US 5,567,422 A (GREENWALD) 22 October 1996, see entire document.                   | 1-50   |
| Y   | US 5,635,207 A (GRINSTAFF et al) 03 June 1997, see entire document.                | 1-50   |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.   |  |  |
| * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"B" earlier document published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed<br>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"A" document member of the same patent family |  |  |
| Date of the actual completion of the international search<br>13 APRIL 2000  |  | Date of mailing of the international search report<br>10 MAY 2000                            |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230   |  | Authorized officer <i>John P. Nutter</i><br>NATHAN M. NUTTER<br>Telephone No. (703) 308-0661 |

## INTERNATIONAL SEARCH REPORT

Internat. : application No.  
PCT/US00/02608

### A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C08G 63/48, 63/91; C12N 11/02, 11/04, 11/06, 11/08; G01N 33/544, 33/545, 33/546, 33/549

### A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

525/54.1; 530/812, 815, 816, 817; 435/177, 180, 181, 182; 436/531, 532, 533, 534, 535